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PROCEEDINGS OF THE ROYAL SOCIETY.

SERIES B.—BIOLOGICAL SCIENCES.

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Experimental Distortion of Development in Amphibian Tadpoles. *Part II.*

By D. E. SLADDEN (Zoology Research Department, Imperial College of Science).

(Communicated by E. W. MacBride, F.R.S.—Received May 18, 1932.)

Introduction.

A previous paper (Sladden, 1930) contained an account of certain experiments carried out with the eggs of the common frog (*Rana temporaria*). The experiments were performed with the object of testing the results obtained from similar ones by Tornier (1908). Tornier states that cane sugar in tap-water brings about a reduction of the amount of oxygen present in solution, and that frogs' eggs subjected to sugar solutions of various concentrations become "weakened"—presumably through lack of oxygen—and give rise to abnormal larvæ. He then goes on to compare these abnormalities with the abnormal "fancy" races of goldfish, assuming the origin of these fancy fish to be due to a deficiency of oxygen in early stages of development.

Working on Tornier's assumption that cane sugar would act as an absorbent of any oxygen present in a given solution, frogs' eggs were subjected to a 10 per cent. solution of sugar in tap-water for a period of 4 hours; 24 hours after fertilisation. Most of the eggs thus treated gave rise to apparently normal larvæ, a certain percentage of the eggs failed to develop (these may have been unfertilised) but in a few cases the resulting larvæ showed various types of abnormality which became recognisable externally at two quite distinct stages in their development and can therefore be placed into two categories described as "early" and "late" abnormalities.

(1) Larvæ abnormal at the time of hatching. These without exception died after a short period of larval life, that is before the second type of abnormality commenced to appear. These early abnormalities showed a type of defect presumably homologous to that described by Tornier as the distended body-cavity, a characteristic peculiar to certain breeds of fancy goldfish.

(2) Larvæ apparently normal at the time of hatching. These proceeded to develop in the usual way until the stage was reached at which the hind limbs first appear externally, about 8-10 weeks after the time of hatching. At this stage some of the larvæ developed a marked lateral bend at the base of the tail; in others, as the hind limbs developed, either one or both limbs were observed to be stiffened (due possibly to a muscular defect in the articulation of the femur with the tibio-fibula) and as a result the affected limb or limbs projected at right-angles to the main axis of the body. This defect rendered the limbs concerned practically functionless, except as a means of support. Sometimes larvæ appeared with one hind limb, either on the right or left side of the body, entirely absent. The limb thus restricted in its growth was usually that which would normally lie in the angle between the body-wall and the curved surface of the tail on the convex side of the tail-bend.

This entire suppression of a limb was always preceded by a bend at the base of the tail, although in the majority of cases the limb managed to develop to normal proportions and function in spite of the constriction caused by the bend. Those tadpoles having only the bend at the base of the tail, gave rise at the end of metamorphoses to young frogs showing various forms of sacral distortion.

These late abnormalities developed from among the larvæ that were regarded as normal at the time of hatching and were separated from the early abnormalities by an interval of several weeks. Therefore the two sets of abnormalities were quite distinctly separated from each other both by the type of defect and the stage in development at which the defect appeared.

Since the publication of the previous paper (1930), Sir Henry Dale suggested to me that it would be possible to confirm Tornier's theory of lack of oxygen by introducing into tap-water containing frogs' eggs either hydrogen or nitrogen, thereby replacing the oxygen by an asphyxiating gas. This suggested experiment has been subsequently carried out, hydrogen being used, owing to the difficulty of obtaining chemically-pure nitrogen.

In addition to the hydrogen experiment the present paper contains the particulars and results of numerous experiments conducted during the past 2 years in connection with the effects of p_{H_2} variation on the development of

frogs' eggs. Further sugar experiments have been carried out, though it is now thought probable that the results obtained from the use of sugar are due to osmosis rather than lack of oxygen as previously supposed.

Frogs' eggs have also been subjected to solutions of boiled water in ordinary tap-water as another possible means of reducing the oxygen content of a solution, but so far these experiments have not given rise to particularly significant results.

Hydrogen Experiment.

For this experiment the eggs of one pair of frogs were used. These eggs were artificially fertilised, divided into three batches, numbered 1-3 (Table I) and placed in tap-water for 24 hours. The apparatus for the experiment consisted of a cylinder of hydrogen, four glass vessels of equal dimensions, each fitted with a rubber stopper. These were connected with each other and with the hydrogen cylinder by means of tubes fitted as bubblers (see diagram), fig. 1. The first two vessels, numbering from the hydrogen cylinder, contained only tap-water, the other two tap-water plus eggs. By this arrangement the hydrogen passed through two vessels of tap-water before coming in contact with the eggs. Stop-cocks were used to equalise the amount of hydrogen passing to the two batches of eggs.

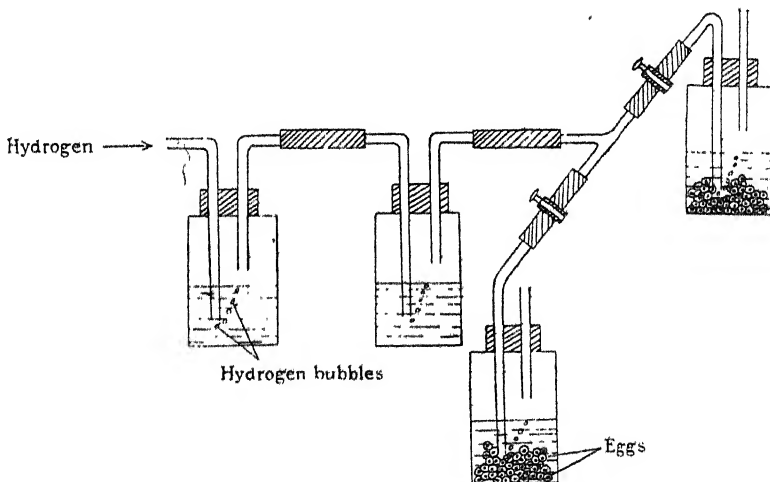


FIG. 1.—Apparatus used in hydrogen experiment.

Twenty-four hours after fertilisation the two batches of eggs were introduced into the two vessels intended for them and hydrogen bubbled through. After 2 hours one vessel containing eggs was disconnected from the apparatus, its

contents removed and transferred back to tap-water. One hour later, after a period of 3 hours, the other egg-containing vessel was disconnected, and its contents treated in the same way as the first batch of eggs. The two batches of eggs now continued their development under the same conditions as the control, batch 3.

Table I contains the results of the hydrogen experiment. As in all experiments so far conducted the resulting abnormal larvæ can be segregated into two quite definite types, according to whether the defects become evident at an early or late stage in the larval development. In the early stage, the per-

Table I.- Results of Hydrogen Experiments.

	Normal larvæ/ total number eggs at hatching, per cent.	Abnormal larvæ/ total number eggs at hatching, per cent.	Dead eggs/total number eggs at hatching, per cent.	Abnormal larvæ/ normal larvæ at hatching, per cent.	Normal larvæ May 28/ number normal larvæ at hatching, per cent.	Abnormal larvæ/ normal larvæ on May 28, per cent.	Dead normal larvæ/ normal larvæ on May 28, per cent.	Meta- morphosed normals/ normal larvæ on May 28, per cent.
D1. Hydrogen for 2 hours	94.6	1.2	4.2	1.3	55.1	3.7	43.1	53.2
D2. Hydrogen for 3 hours	92.8	1.0	6.2	1.1	78.8	5.3	29.2	65.4
D4. Hydrogen con- trol	97.0	0.3	2.7	0.3	59.8	3.7	39.8	56.4

centage of normal and abnormal larvæ and undeveloped eggs, is taken of the total number of eggs in each batch at the time of hatching, with the additional ratio, abnormal to normal larvæ at the same stage. In the late stage the percentages are based on the number of larvæ, all apparently normal, living on May 28, approximately 9 weeks after hatching, when only such larvæ remain. Therefore the second percentage of abnormal larvæ is not an additional percentage to that taken at the time of hatching, as at the early stage the second type of abnormal larvæ had not commenced to appear.

The significant results from the hydrogen experiment included in the table will be dealt with as follows—Batch 1, subjected to hydrogen for 2 hours, gave rise to the greatest number of larvæ from any one batch showing abnormalities at the early stage. At the late stage the percentage of abnormalities in the same batch shows a decrease in numbers when compared with the number of abnormal larvæ in the second batch at a corresponding stage in development, while the number of deaths among the normals up to metamorphoses has increased.

Batch 2, subjected to hydrogen for 3 hours, gave rise to comparatively few early abnormalities, but this batch contained more undeveloped eggs than the previous one; these may have been unfertilised, or unable to survive the longer subjection to hydrogen and so died during the experiment. The number of abnormal larvæ at the late stage shows an increase when compared with the abnormal larvæ in the first batch at a corresponding stage in development. Therefore in batch 1, the number of early forms predominates over the late ones, while in batch 2 this is reversed.

In the case of the control it will be noticed that a considerable number of the larvæ show abnormalities at a late stage in development. This is contrary to the results obtained from other experiments, notably the sugar ones, where the percentage of abnormalities in the control was almost negligible. A possible explanation of this will be considered later.

The results of the above experiments are not conclusive, but tend to indicate that the early abnormalities arising from this and other experiments are not due to lack of oxygen as previously assumed. How far osmosis or p_H variation influences the developing eggs and subsequently the larvæ has yet to be determined.

p_H Experiment.

In 1930 preliminary experiments were carried out in connection with p_H . HCl was used to lower the p_H and KOH to raise it. The eggs from one pair of frogs were artificially fertilised, divided into three batches, numbered 1 to 3, Table II, and placed in tap-water. Twenty-four hours later, batch 1 was transferred to previously tested tap-water, p_H 6.0. Batch 2 was the control in tap-water, p_H 7.5 and batch 3 was subjected to tap-water p_H 9.0. The vessels used to contain the eggs during the time they were subjected to acid and alkaline solutions were fitted with rubber stoppers and kept closed. The

Table II.—Results of p_H Experiments, 1930.

	Normal larvæ/total number eggs at hatching, per cent.	Abnormal larvæ/total number eggs at hatching, per cent.	Dead eggs/total number eggs at hatching, per cent.	Abnormal larvæ/total larvæ at hatching, per cent.	Normal larvæ June 26/ number normal larvæ at hatching, per cent.	Abnormal larvæ/normal larvæ on June 26, per cent.
1. p_H 6.0	92.6	3.3	3.9	3.6	47.0	25.4
3. p_H 7.5	89.5	3.5	7.0	3.9	67.3	0.0
2. p_H 9.0	88.0	3.4	8.5	3.9	20.6	5.7

contents of the vessels were stirred at intervals during the course of the experiment. After a period of 4 hours the eggs were transferred back to normal conditions, *i.e.*, p_H 7.5, to continue their development.

The following year these preliminary experiments were succeeded by two parallel experiments of a similar nature. The eggs used were from two pairs of frogs, A and B, Table III, and this time only acid solutions were used. The eggs from both pairs were artificially fertilised, divided into four batches, numbered 1 to 4, Table III, and placed in tap-water. Twenty-four hours later the eggs from pair A, batches 1 to 4, were transferred to previously tested tap-water, p_H 5.0, 6.0, 6.5 and 7.5 respectively. The control being p_H 7.5, as before. The vessels were kept closed and their contents stirred at intervals during the time of the experiment, 4 hours later the eggs were transferred back to normal conditions. The eggs of the second pair, B, were subjected to exactly similar conditions throughout the experiment.

Table III. —Results of p_H Experiments, 1931.

	Normal larvæ/ total number eggs at hatching, per cent.	Abnormal larvæ/ total number eggs at hatching, per cent.	Dead eggs/total number eggs at hatching, per cent.	Abnormal larvæ/ total larvæ at hatching, per cent.	Normal larvæ May 28/ number normal larvæ at hatching, per cent.	Abnormal larvæ, normal larvæ on May 28, per cent.	Dead normal larvæ normal larvæ on May 28, per cent.	Meta- morphosed normals/ normal larvæ on May 28, per cent.
A1. p_H 5.0	85.6	2.6	11.8	3.1	26.0	5.9	5.9	88.2
A2. p_H 6.0	86.4	0.7	12.9	0.8	53.1	8.3	42.5	49.2
A3. p_H 6.5	84.0	1.4	14.6	1.6	46.2	7.9	18.1	74.0
A4. p_H 7.5	87.2	0.9	11.9	1.0	47.9	6.7	28.5	64.8
B1. p_H 5.0	81.3	0.6	18.1	0.8	73.2	44.9	26.4	28.6
B2. p_H 6.0	82.0	0.2	17.8	0.3	88.6	43.3	28.8	27.9
B3. p_H 6.5	82.2	0.8	16.9	1.0	87.6	38.4	26.7	34.9
B4. p_H 7.5	86.2	0.2	13.6	0.3	75.2	20.2	41.2	38.6

These two series of experiments were carried out with the object of determining what effect, if any, p_H changes would have on developing frogs' eggs and subsequently the tadpoles. In the first series (1930) the results of the experiments are given as far as they are known, for at the late stage only the ratio abnormal to normal larvæ, living on June 26, was recorded. These results are as follows:—At the early stage only a few abnormalities occurred, the greatest number from among the control. The late stage, however, shows a high percentage of abnormal larvæ from batch 1, p_H 6.0. The predominant defect among the larvæ at this stage took the form of a bend at the base of

the tail; a defect common among abnormal larvæ from other experiments; giving rise subsequently to sacral distortion. Both batches 1 and 2 gave rise to a few larvæ which at the late stage developed distended body-cavities, a defect frequently occurring in abnormal larvæ at the time of hatching, but one which had so far not appeared in larvæ at the late stage.

As the preliminary p_H experiments just described gave rise to apparently significant results, especially as with batch 1, in which the tap-water was rendered slightly acid (p_H 6.0), the result was distinctly positive, it was decided to conduct further experiments on these lines. Therefore in 1931, two parallel experiments were carried out, concentrating on decreasing the p_H of tap-water solutions by means of HCl as before. The tabulated results of this experiment are constructed on the same general principle as those of the foregoing experiment, and, except for the additional records of dead and metamorphosed normal larvæ at the late stage, the two tables are comparable. No conclusions are being drawn from the results obtained from pair A on account of the high mortality among the larvæ before the late abnormalities commenced to appear.

The results as in the previous year tend to indicate that eggs subjected to tap-water, the p_H of which has been artificially lowered, give rise to only a limited number of early abnormalities, but at the late stage there is a marked increase in the number of abnormal larvæ from the eggs of both pairs of frogs. The eggs of the second pair, B, however, gave rise to a greater number of abnormalities at this stage as compared with those of pair A at the same stage in development, and with a corresponding increase in the percentage of abnormal larvæ occurring in the control.

As in the previous p_H experiments, the principal type of defect becoming evident at the late stage was the bend at the base of the tail, giving rise to sacral distortion in the newly metamorphosed frogs. Very few cases of stiffened or otherwise defective limbs occurred as a result of this experiment and none of the larvæ developed distended body-cavities at this stage.

Sugar Experiments.

In addition to the hydrogen and p_H experiments just described, a further series of sugar experiments have been carried out, partly in an attempt to determine whether the action of sugar on the developing eggs was purely osmotic, and whether the stage in development at which the stimulus was applied would have any bearing on either the type or percentage of subsequent abnormalities.

In 1930, 10 per cent. solutions of cane sugar in tap-water were applied to batches of frogs' eggs at 3, 6, 24, and 48 hours after fertilisation, over the usual period of 4 hours. The eggs used were from three pairs of frogs (C, E and F, Table IV). Eggs from pair C were directly fertilised and from pairs E and F artificially fertilised. As far as possible the eggs were subjected to the same treatment, which was as follows: eggs divided into two or more batches, numbered 1, 2, etc., and kept in tap-water until required for the experiment being transferred back to normal conditions after treatment. The eggs of a fourth pair, D, were subjected to 10 per cent. solution of sugar in distilled water 24 hours after fertilisation for 4 hours (D3). (Control batch D4, Table IV, for further particulars consult table.)

Table IV.—Results of Sugar Experiments, 1930.

	Normal larvæ/total number eggs at hatching, per cent.	Abnormal larvæ/total number eggs at hatching, per cent.	Dend eggs/total number eggs at hatching, per cent.	Abnormal larvæ/normal larvæ at hatching, per cent.	Normal larvæ, June 26, number normal larvæ at hatching, per cent.	Abnormal larvæ/normal larvæ on June 26, per cent.
C1. 10 per cent. sugar solution at 3 hours	81·6	5·6	12·6	7·0	61·7	7·1
C2. 10 per cent. sugar solution at 6 hours	77·8	5·5	16·7	7·1	63·5	7·7
F1. 10 per cent. sugar solution at 24 hours	24·9	1·5	72·9	6·0	35·7	6·9
F4. Control	43·4	0·6	55·9	1·5	42·2	0·0
D3. 10 per cent. sugar in distilled water at 24 hours	84·6	3·2	12·1	3·8	72·6	1·7
D4. Control	86·3	1·3	12·3	1·6	6·0	0·0

In 1931 the progeny of one pair of frogs was used, Table V. The eggs were artificially fertilised and divided into four batches numbered 1 to 4. Batches 1 to 3 were subjected to 10 per cent. sugar solution in tap-water 24 hours after fertilisation, for periods of 4, 6 and 8 hours respectively; batch 4 was kept as the control.

So far treatment had been confined to eggs 24 hours after fertilisation, *i.e.*, at the end of segmentation. The effect was now tried of applying the stimulus to the eggs at 3 and 6 hours after fertilisation, at the 24-hour stage as before and at the 48-hour stage. These times would correspond approximately to the following stages: 1 hour before the first cleavage, at the 32-cell stage, at the end of segmentation and at the beginning of gastrulation. The results of this experiment of 1930 (Table IV), are tabulated on the usual plan and comparable with the results of other experiments.

Subjecting the eggs to sugar solutions at the 3 and 6-hour stages gave rise to numerous early abnormalities, the majority of these possessing distended body-cavities. These larvæ die shortly after hatching. At the late stage, in addition to the curvature of the tail, a large number of the larvæ developed stiffened limbs. Only the hind limbs were affected, either the right or the left or in rarer cases both. As before, sugar applied at the 24-hour stage gave rise to larvæ at the late stage of development with the bend at the base of the tail, but only a few cases of stiffened limbs occurred.

The results obtained when sugar was applied 48 hours after fertilisation are omitted from Table IV for the following reason. The reaction of the eggs to sugar applied at this stage was the formation of one or more spherical excrescences over their surfaces, which in section appeared to be due to proliferation of the ectoderm. Without exception these eggs died during the course of the experiment, or at the most failed to develop beyond the gastrula stage.

Distilled water, used instead of tap-water in making up the solutions, does not appear to have any effect on the results obtained from either experimental or control batches, Table IV, D3 and D4.

The short period each year during which it is possible to obtain copulating frogs limits the number of experiments that can be conducted in one season, and for this reason the second series of experiments on these lines had to be postponed until the following year. This second series consisted of applying 10 per cent. sugar solution at only one stage in development, namely, 24 hours after fertilisation, but over longer periods, Table V.

Table V.—Results of Sugar Experiments, 1931.

	Normal larvæ/ total number eggs at hatching, per cent.	Abnormal larvæ/ total number eggs at hatching, per cent.	Dead eggs/total number eggs at hatching, per cent.	Abnormal larvæ normal larvæ at hatching, per cent.	Normal larvæ May 28/ number normal larvæ at hatching, per cent.	Abnormal larvæ/ normal larvæ on May 28, per cent.	Dead normal larvæ/ normal larvæ on May 28, per cent.	Meta- morphose normals/ normal larvæ on May 28, per cent.
C1. 10 per cent. sugar solution for 4 hours	85.6	1.2	13.2	1.4	61.6	19.4	23.8	56.7
C2. 10 per cent. sugar solution for 6 hours	94.7	3.8	1.5	4.7	15.0	19.3	9.6	71.1
C3. 10 per cent. sugar solution for 8 hours	95.9	1.2	2.9	1.2	51.5	20.7	19.1	60.1
C4. Control	96.9	0.2	2.9	0.2	55.9	9.4	19.6	70.9

The results at the early stage are not significant and can be passed over. At the late stage, the most important fact appears to be that when compared with the first series of experiments at a corresponding stage, the number of larvæ which occurred with defective limbs was almost negligible, although the abnormal larvæ showed various degrees of tail curvature and sacral distortion.

From among the larvæ in batch I of this series, sugar applied for 4 hours, several cases occurred in which either the right or left limb was duplicated. Both the limbs were slightly reduced in size when compared with the corresponding limb of a normal larva at the same stage in development. With regard to position, the two limbs on the one side were either superimposed dorso-ventrally, the ventral one always the smaller, or the latter projected at an angle to the larger dorsal one. In one larva the two limbs were fused as one over the length of the femur, and then separated into two perfectly formed limbs. These double limbs are the first instances of doubling of any organ that have so far occurred.

The defect is probably due to a division of the developing limb bud. Several recognised types of fancy goldfish, such as the Demekin and Oranda Shishigashira, have the caudal fin completely double, due to separation of the tail rudiments during development by the intrusion of swollen yolk.* In an 11 mm. tadpole there is a quite definite aggregate of cells in the developing limb bud, while the gut still retains large quantities of yolk.

As previously stated, batches of frogs' eggs have been subjected to solutions of tap-water, 50 per cent. of which had been previously boiled, and allowed to cool in a plugged flask. The object of this experiment was to reduce the amount of free oxygen in tap-water by boiling. The results of this experiment at both early and late stages are recorded in Table VI.

Table VI.—Results of Boiled Water Experiments, 1930.

	Normal larvæ/total number eggs at hatching, per cent.	Abnormal larvæ/total number eggs at hatching, per cent.	Dead eggs/total number eggs at hatching, per cent.	Abnormal larvæ/normal larvæ at hatching, per cent.	Normal larvæ June 26/ number normal larvæ at hatching, per cent.	Abnormal larvæ/normal larvæ on June 26, per cent.
F2. 50 per cent. boiled in 50 per cent. tap-water at 24 hours	40.7	0.8	58.4	2.1	59.1	3.5
F4. Control	43.4	0.6	55.9	1.5	42.2	0.0

* Professor MacBride refers to this in a paper on "The Work of Tornier," published in the "Eugenics Review" of 1924.

It was decided to include the somewhat insignificant results of this experiment in the tables, though they do not appear to be of particularly fundamental importance.

Discussion.

The comparatively high percentage of abnormal larvæ occurring among the controls of all experiments at both early and late stages in development tends to indicate the presence of a so-far undetermined factor having a fundamental influence on the developing eggs. General conclusions indicate that some factor or factors are at work which have a greater influence on the development of the eggs than either the action of sugar or p_{H} variation, and thereby obscure the results of the experiments.

From observations made on a large series of experiments of various kinds involving the rearing of many thousands of frog tadpoles, it appears very probable that this unknown factor comes into play as a result of overcrowded conditions. (According to Tornier, the races of fancy goldfish owe their origin to the overcrowded and unhealthy conditions in which they are bred.)

If larvæ are subjected to overcrowded conditions, the oxygen content of the water will be considerably reduced, while the amount of carbon dioxide and nitrogenous products are automatically increased. As development under these conditions proceeds, the stronger and more vigorous larvæ will rapidly increase in size at the expense of the weaker ones, so that it soon becomes possible to grade the larvæ into small, medium, and large forms. If the larvæ are not separated into the three grades before the critical period in development, when the change from vegetable to animal diet takes place, only the larger forms will be sufficiently well nourished to metamorphose normally. The medium sized forms, weakened through lack of food and oxygen, will continue to develop, but it is from among these larvæ that the greater number (but by no means all) of the late abnormalities occur. These larvæ, both normal and abnormal, after a somewhat retarded larval development finally metamorphose several months later into undersized frogs. These latter are extremely difficult to rear through to the adult stage. In the case of the small larvæ, the majority of these only live for a few weeks after hatching, about three or four, until the yolk in the gut is finally all absorbed, when, the supply of vegetable diet available being quite inadequate they gradually decrease in size and finally die.

Summarising these somewhat inconclusive results, it would seem that the effect of sugar is not the removal of oxygen as previously assumed, but very

possibly osmotic action and is responsible for the early abnormalities in particular, while p_{H_2} decrease may be responsible for the late ones.

It now seems possible, however, that overcrowding may have seriously affected all the previous results. In order to test this hypothesis a series of experiments is now being designed which will involve only a limited number of larvæ, so that the possibility of overcrowded conditions will be practically eliminated.

I am very grateful to Sir Henry Dale, Sec. R.S., for his valuable suggestion and also to Mr. J. Gray, F.R.S., whose advice was followed in constructing the apparatus used in connection with the hydrogen experiment.

I wish to express my sincere gratitude to Professor E. W. MacBride, F.R.S., for affording every possible facility to carry out this research, and to Mr. H. R. Hewer, M.Sc., whose advice and assistance, always freely given, has proved invaluable on every occasion. Thanks are also due to Mr. T. L. Green for his help with the practical work these experiments involved.

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Studies in Insulin. 1.—Kataphoresis of Insulin alone and in presence of glucose with some reference to the question of an Insulin-Glucose reaction in vitro.

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Insulin has been the subject of innumerable investigations on account of its physiological importance in the control of carbohydrate metabolism. Few of the researches on this hormone, however, have dealt with aspects other than purely physiological ones. The physico-chemical behaviour of insulin appears to the authors to merit attention on the grounds that such characteristics may play a part in its control of the blood-sugar level in the animal organism.

The present series of papers is the outcome of an attempt to determine the character of the surface charge and of other physico-chemical properties of insulin protein and to correlate the results thus obtained with its physiological activity. It was considered also that the titration of insulin against acids and alkalis would throw some light on the possible association of basic and acid groups with the centres responsible for its remarkable activity. This latter theme is the subject of a separate communication.

Insulin is a well-defined member of the protein group and hence its surface charge would be expected to conform with that of the other members of this class of substances, *i.e.*, it would be dependent on the $[H^+]$ of the surrounding medium when solutions or, to be more accurate, sols in aqueous media are considered.

The exact category of proteins into which insulin may be placed is not immediately evident. Abel and co-workers (1927), who first isolated the hormone in a crystalline condition, recorded an empirical formula of $C_{45}H_{75}O_{17}N_{11}S$, and determined the proportions of its constituent amino-acids. This and other evidence of a chemical nature was a sure indication that the structure of insulin was that of a high polypeptide.

With regard to its physical properties, insulin is insoluble in water but readily dissolves on addition of small amounts of acid or alkali. Adjustment of the

reaction of a solution so obtained to p_H 5.5-5.7 is accompanied by a reprecipitation of the insulin in an isoelectric condition. Acid or alkali effects solution owing to the formation of the acid or basic salt according to the generally accepted theory of amphoteric proteins. These facts together with the insolubility in organic solvents, non-permeability to membranes such as parchment, and behaviour towards high concentrations of salts as ammonium sulphate verify its protein-like nature. Insulin is perhaps best classified as a globulin on account of its insolubility at the isoelectric point. Sjögren and Svedberg (1931), by determination of the rate of sedimentation during ultracentrifugation have concluded that the molecules of crystalline insulin are, with regard to mass, shape and size, almost identical with those of ovalbumin and Bence-Jones protein, the molecular weight being 35,100 over the range of p_H stability 4.5-7.0.

The isoelectric point of pure insulin in aqueous solution has been fairly well established by the work of Abel *et al.* (*loc. cit.*) who deduced a value of p_H 5.5-5.6 from the solubility and from the readiness with which crystallisation occurs from buffered pyridine-brucine solutions. The most valued criterion of the isoelectric condition, namely, the determination of that reaction at which the surface charge is to a minimum, has so far not been utilised. In addition to the determination of the isoelectric point, kataphoresis affords a method of investigating the question of an *in vitro* glucose-insulin reaction. Although our experiments indicate a physico-chemical interaction occurring between glucose and insulin in alkaline solutions, the existence of any reaction capable of providing a physiological parallel has not been demonstrated. This is in agreement with the majority of workers who have studied this problem from the viewpoint of the behaviour of the sugar.

It should be noted at this juncture that Keller and Diekhorn (1926), using a rather crude type of apparatus, found that both insulin and glucose migrated to the anode at the reaction of tissue fluids. The possibility of a substance such as glucose possessing the electrically charged surface peculiar to small particles appears questionable.

Krüger (1929) by means of diffusion experiments failed to demonstrate the occurrence of complex-formation between glucose and proteins such as ovalbumin at neutral reaction. This, however, does not exclude the possibility of such a phenomenon in the instance of insulin, a hormone which, as Wierzychowski (1926) has commented, appears to have been specifically set up for glucose.

EXPERIMENTAL.

Materials.

Insulin Hydrochloride.—This material was prepared in the laboratories of Messrs. Boots Pure Drug Co., Ltd. It was a white amorphous powder and when tested on the "cross-over" method of Marks (1925) showed a potency of 25 international units per milligram. Neglecting earlier claims for the potency of pure insulin the generally accepted view at the present time is that the hormone in its purest condition has an activity of 24–25 units per milligram. Thus, Culhane, Marks, Scott and Trevan (1927) carried out extensive determinations employing various biological methods of assay on crystalline material and found that as an average of all the results obtained one international unit was equivalent to 1/24 mg. Admittedly other workers have claimed the production of a material of 40 or more units per milligram (Dingemans *et al.*, 1927, 1928; Peek, 1929; Dirscherl, 1931). Such claims, however, have not been satisfactorily sustained (Jensen and De Lawder, 1930). It was therefore considered that the sample used represented in the light of present knowledge "pure" insulin. Incineration of 20 mg. in a platinum dish left no perceptible or weighable ash.

Glucose.—Glucose monohydrate was twice recrystallised from 80 per cent. aqueous alcohol and dried over phosphorous pentoxide in a vacuum. Stock solutions (1.0 per cent.) prepared from this material were tested for reducing power and rotation and found to have a glucose content of 0.99–1.005 per cent.

Buffer.—A 0.2 M solution of the "Universal Buffer" of Prideaux and Ward (1924) was prepared in distilled water.

Gold Sol.—A stock solution was prepared by Bredig's method by passing an arc between two gold wires in 0.0005 N-HCl (Beans and Eastlack, 1915). Colorimetric comparison with standard gold solutions reduced by the phosphorus-ether method indicated an approximate concentration of 0.05 per cent. in colloidal gold.

Apparatus.—The apparatus previously described (1929, 1930) was modified according to the plan adopted by Kruyt and van der Willigen (1928) a description of which has already been given (Howitt and Prideaux, 1930). For this purpose secondary electrodes were introduced into the limbs of the main U-tube. These secondary electrodes consisted of thin rubber-covered copper wires at the end of which were soldered small platinum spirals. The main electrodes, which were as previously described, were connected directly to the main supply and a voltmeter. The secondary electrodes were connected in

series with secondary batteries and a potentiometer so that a potential of 0-180 volts could be obtained, whilst a unipivot galvanometer fitted with a "range multiplier" shunt (Cambridge Scientific Instrument Company) in series and a voltmeter in parallel indicated the current and potential respectively.

Kruyt (*loc. cit.*) has shown that for this type of apparatus the velocity of electrophoresis is given by the relation

$$v = \frac{w_1 h_1 + w_2 h_2}{ZE}$$

where

w_1 = displacement downwards in centimetres
 w_2 = displacement upwards in centimetres
 h_1 = total length of buffer column.
 h_2 = total length of sol column.
 E = potential across secondary electrodes.

The values of h_1 and h_2 for the apparatus used were 82 mm. and 192 mm. respectively. Expressing w_1 and w_2 in centimetres, h_1 and h_2 in millimetres, Z in seconds and E in volts, the value for v are given in μ per second per volt per centimetre when multiplied by 1000.

In practice it was found that the value of E occasionally varied by 1-3 volts during the course of an experiment. An average value was then taken.

Preparation of Solutions for Electrophoresis.

(a) *Insulin-Gold Sol.*—10 c.c. of a 0.10 per cent. solution of the insulin hydrochloride were placed in a 50 c.c. graduated flask and 5 c.c. of the 0.2 M buffer solution added. The reaction of such a mixture was p_H 3.45. Acid or alkali (0.1 N-HCl or NaOH) was then added to give the desired reaction, a close approximation to which could be calculated from the equation applicable to this particular buffer mixture. This was followed by 10 c.c. of the gold sol and the resulting solution was made up to 50 c.c.

Thus, the final concentrations were 0.02 M in buffer, 0.02 per cent. in insulin and 0.01 per cent. in gold. Despite the low concentration of insulin it was found that over a certain range of p_H , namely, 4.5-6.5 the protein was iso-electrically precipitated within a few minutes. As will be readily understood, the presence of the gold sol contributes towards the instability of the protein micellæ. Such precipitates were readily peptised by addition of acid or alkali. At reactions bordering on this range the complex sols exhibited a partial degree of instability and determinations had to be carried out within a few hours in order to anticipate this flocculation.

(b) *Insulin-Glucose-Gold Sol.*—The solutions were prepared as for (a) excepting that 5.0 c.c. of 1.0 per cent. glucose solution were added before making up to 50 c.c., giving a final concentration of 0.1 per cent. in glucose.

(c) *Incubated Insulin-Gold Sol.*—Solutions were prepared as for (a) and then incubated at 37° C. for 24 hours before determining the electrophoretic velocities.

(d) *Incubated Insulin-Glucose-Gold Sol.*—Solutions were prepared as for (b) and then incubated at 37° for 24 hours before use.

(e) *Buffer Solutions.*—To 5 c.c. of the 0.2 M—buffer solution acid or alkali were added to give the desired reaction followed by dilution to 50 c.c. The p_H of the final solution was determined so as to ensure agreement with that of the corresponding insulin-gold sol.

Distilled water free from CO_2 was used throughout for the preparation of these solutions, the $[H^+]$ of which were determined by the hydrogen electrode and saturated calomel half-cell.

Electrophoresis.

The determinations were carried out at room temperature (t°), the results being corrected to any desired temperature (x°) by the viscosity ratio η_t/η_x . Migration was observed for periods of 30–75 minutes during which the velocity remained quite constant except in a few instances when the experiment was repeated.

Before consideration can be given to the actual experimental results, it is of considerable importance to establish the following two facts :—

- (a) That addition of gold sol to an insulin solution has no effect on the activity of the latter.
- (b) That the migration of the gold sol is an absolute criterion of the migration of the physiologically active principle.

In order to test the first factor, insulin and gold sol solutions identical with those used in the kataphoretic experiments were prepared. These solutions (at p_H 3.5, 7.95 and 8.6 respectively) were tested at 5 units per cubic centimetre (the strength calculated from the insulin content of 0.02 per cent.), using for each test six rabbits which has previously been standardised on the international insulin standard. The strengths indicated were 4.9, 4.0 and 5.15 units per cubic centimetre respectively. It was concluded that the solutions actually used for kataphoresis contained the insulin in a physiologically active form.

With reference to the second factor, consideration has previously been given to the fact that a gold sol alone will migrate when situated in an electric field (Prideaux and Howitt, 1932). As in previous experiments the colourless fluid in the bore of the tap from which the gold had moved in a downward direction was removed by a fine pipette and tested for protein by picric and phosphotungstic acids. The results were negative in every instance and indicated that the protein was moving with the gold. The close association of gold sol and insulin was confirmed in two ways. Firstly, the blood-sugar lowering capacity of such insulin-gold sols (p_H 3.6 and 8.5) was approximately compared with that of similar solutions which had electrophoretically migrated above the bore of the tap and which had been removed by means of a fine pipette; owing to the extremely small amount of solution thus obtained the tests could be carried out with only three rabbits for each assay. For each of the two "migration fluids" the same three rabbits were used for the testing of the fluid itself and of the original fluid before migration: further the animals were tested on an insulin standard. The same dose (0.5 units per kilogram body weight) was injected, the assumption being made that the migration fluid had retained all its original activity (*i.e.*, 5 units per cubic centimetre). Calculated for the insulin standard as 100 the results were:—

p_H 3.6	Migration fluid	96.5 per cent.
		Original fluid	94 per cent.
p_H 8.5	Migration fluid	94 per cent.
		Original fluid	90 per cent.

Thus, allowing for the error inherent in an insulin assay where so few rabbits are used, the migration fluid has retained all its original activity. Secondly, the colourless fluid from which the insulin-gold complex had migrated was shown to be devoid of physiological activity. This test, which was considered of greater importance than that on the migration fluid owing to the bearing on the question of the homogeneity of the insulin preparation used, was carried out on mice according to the method of Hemmingsen and Krogh (1926).

The residual fluids (from insulin-gold sol preparations of p_H 3.46 and 8.86) were diluted $\times 12$ whilst the original preparations, diluted $\times 100$, served as controls. Thus the potency of the fluids would be 0.42 units per cubic centimetre if no active material had migrated whilst that of the controls was 0.05 units per cubic centimetre. The dose injected was 1/50 unit per 20 gm. body weight for the original preparations and for the standard insulin solution whilst the equivalent of approximately eight times this was used in the case of the residual fluids.

Convulsions.

Residual fluid (p_H 3.46; diluted $\times 12$)	3/20
Original preparation (p_H 3.46; diluted $\times 100$)	10/20
Standard insulin solution (0.05 units per cubic centimetre)	10/20
Residual fluid (p_H 8.86; diluted $\times 12$)	0/20
Original preparation (p_H 8.86; diluted $\times 100$)	10/20
Standard insulin solution (0.05 units per cubic centimetre)	12/20

A consideration of the above results indicates that within the limits of experimental error, practically all the active material had migrated. The slight response by the p_H 3.6 residual fluid (which indicates a retention of 1/28 of the original activity) is probably due to a small amount of the active "migration fluid" being swept into the pipette during the withdrawal of the residual fluid. This contamination is extremely difficult to avoid owing to the surface of separation of residual and migration fluids being but 2-3 mm. distant from the orifice of the pipette. The pipette consisted of a fine glass tube (at the open end it was about 1 mm. in diameter) with the open end curved upwards and with a small screw clip on the teat at the reservoir end so that liquid could be drawn into the pipette with a minimum of disturbance.

Confirmation of the retention of physiological activity by insulin in presence of gold sol is also provided.

The above tests (which were carried out by the Pharmacological Department of Messrs. Boots Pure Drug Co., Ltd., Nottingham, under the direction of Mr. W. A. Broom, B.Sc., and through the courtesy of Dr. F. L. Pyman, F.R.S., to whom the authors wish to express their indebtedness) affords an assurance that the movement of the gold-buffer interface is a reliable criterion of the actual movement of the insulin itself.

Table I.—The Electrophoretic Velocities of Insulin.

p_H	Temperature.	w_1 (cm./hr.)	w_2 (cm./hr.)	E (volts)	$v = \frac{(w_1 l_1 + w_2 l_2) 1000}{3600 + E}$ u/sec.	$r \propto \frac{\eta_t}{\eta_{18}}$
	°					
2.84	20	3.9	2.2	140	+1.51	+1.43
3.45	20	4.3	2.5	155	+1.49	+1.42
3.90	19.8	3.15	1.35	147	+0.98	+0.93
4.25	20	2.75	1.5	147	+0.97	+0.92
7.11	17	3.35	2.75	165	-1.35	-1.39
7.86	19.5	3.93	3.07	147	-1.73	-1.66
8.56	16.5	4.3	2.7	165	-1.47	-1.53
9.06	20.5	4.6	3.0	153	-1.73	-1.64

Table II.—The Electrophoretic Velocities of Insulin-Glucose.

p_H	Temperature.	w_1 (cm./hr.)	w_2 (cm./hr.)	E (volts)	$v = \frac{(w_1h_1 + w_2h_2)1000}{3600 \times E} \text{ u./sec.}$	$v = \frac{\eta t}{\eta_{18}}$
	°					
2.82	15	4.5	1.9	160	+1.28	1.38
3.0	16.5	4.8	1.0	160	+1.02	1.06
3.50	14.5	4.1	1.9	165	+1.18	1.29
3.84	13.5	3.7	1.3	168	+0.91	1.03
4.24	19	3.6	1.7	167	+1.03	1.01
6.80	16.5	3.1	2.1	172	+1.06	1.10
7.62	13.5	3.6	2.8	163	+1.42	1.60
8.64	16	4.7	3.8	164	+1.89	1.99
9.45	19	4.9	4.0	164	+1.98	1.93

Table III. —The Electrophoretic Velocities of "Incubated" Insulin.

p_H	Temperature.	w_1 (cm./hr.)	w_2 (cm./hr.)	E (volts)	$v = \frac{(w_1h_1 + w_2h_2)1000}{3600 \times E} \text{ u./sec.}$	$v = \frac{\eta t}{\eta_{18}}$
	°					
7.02	17	3.6	1.95	169	+1.10	1.13
7.90	17	4.4	3.5	165	+1.74	1.78
8.78	17	4.8	3.6	166.5	+1.81	1.86

Table IV.—The Electrophoretic Velocities of "Incubated" Insulin-Glucose.

p_H	Temperature.	w_1 (cm./hr.)	w_2 (cm./hr.)	E (volts)	$v = \frac{(w_1h_1 + w_2h_2)1000}{3600 \times E} \text{ u./sec.}$	$v = \frac{\eta t}{\eta_{18}}$
	°					
3.07	19	3.55	1.35	150	+1.14	1.13
3.90	16.5	3.8	1.90	167	+1.13	1.17
7.03	17	3.6	2.4	169	+1.30	1.34
8.08	17	4.6	3.8	161	+1.91	1.96
8.54	17	5.4	5.2	164	+2.44	2.38

The above values are plotted in graph form in fig. 1.

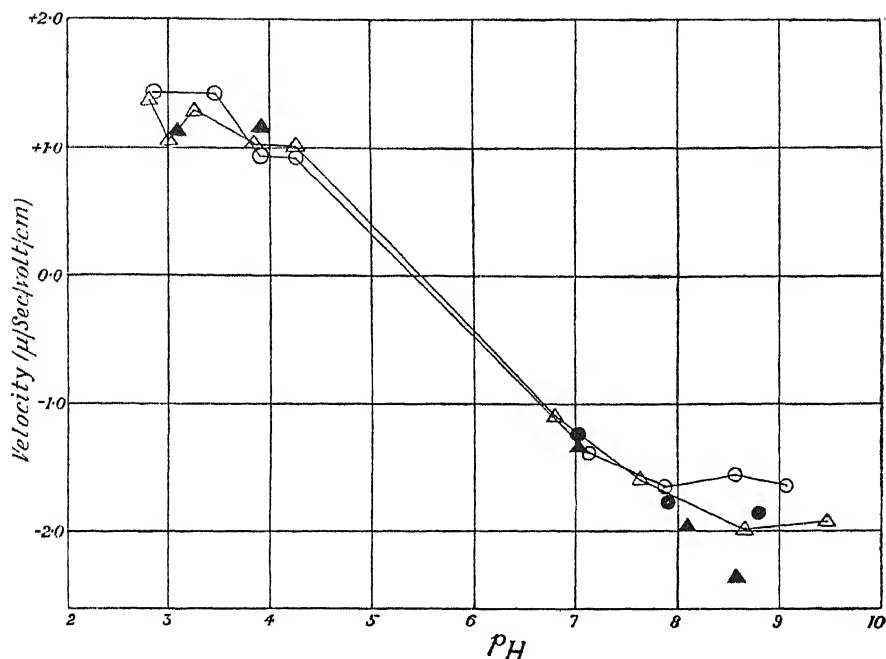


FIG. 1.--Kataphoretic velocities of insulin. ○ Insulin; △ insulin and glucose; ● incubated insulin; ▲ incubated insulin and glucose.

Discussion.

The kataphoretic curve of insulin exhibits a marked flattening out on the alkaline side commencing at a reaction of p_H 7-7.5 and to a smaller extent on the acid side from p_H 4-3.5. It cuts the line of zero velocity at p_H 5.4 which is the isoelectric point. The exactitude of this value no doubt suffers to some extent on account of the difficulty of obtaining migration data at neighbouring reactions. The agreement with values determined by other methods is, however, quite good and it appears that the value p_H 5.4 closely approximates to the true value.

It must be borne in mind that proteins such as insulin, serum globulin, etc., do not possess a clearly defined isoelectric point, but rather do they exhibit an isoelectric zone, *i.e.*, a definite range of p_H over which approximately zero solubility is shown. The acceptance of a definite isoelectric point, however, appears to be feasible when consideration is given to the titration data (Paper II of this series to be published later).

In the presence of glucose the migration velocities within the range of $[H^+]$ of p_H 2.8-7.8 agree closely with the corresponding values for insulin alone;

within the blood range they are almost identical. The slight but sudden drop at p_H 3.0 is perhaps due to experimental errors and any possibility of a correlation with the formation of insulin hydrochloride (reference to which will be found in the succeeding paper of this series) is remote. The main theme of the picture presented by this curve is that at the reaction of the blood and at room temperatures, glucose does not effect any material differences in the surface charge of the insulin micella. The action of insulin on the blood-sugar of the mammal indicates that one molecule of insulin will remove 700,000 molecules of glucose from the blood (Howitt, 1931). Thus, to express this equivalence in the solutions used in the above experiment, a 0.02 per cent. insulin solution would have to be approximately 70 per cent. glucose! Rejecting any hypothesis dependent on stoichiometrical reaction, a preliminary phase of insulin action may be visualised as one of combination between a molecule of insulin and a certain number of glucose molecules. It was expected that this preliminary union, if such did occur, would be indicated by a change in the electrokinetic potential of the insulin micella interface. No such indication is shown by our experimental results. At reactions greater than p_H 7.8 the migration velocities are higher than the corresponding values for insulin alone; it is concluded that at higher degrees of alkalinity glucose has some specific action on insulin. The acidic properties of glucose conceivably play some part in this action for glucose is known to behave as a dibasic acid with dissociation constants, pK'_1 and pK'_2 , at 12.09 and 13.85 (Urban and Shaffer, 1932). Incubation of the glucose-insulin mixtures at 37° for 24 hours serves to emphasize the phenomenon even when allowance is made for the effect of incubation of insulin alone at similar degrees of alkalinity. This effect was at first considered to be due to a loss of physiological activity as a consequence of the alkalinity of the solutions. That this conception is erroneous was clearly indicated by the assay of the activities of such solutions before and after incubation. Thus, a solution at p_H 7.95, tested on six standardised rabbits, had a potency of 4.0 units per cubic centimetres before incubation and, tested on the same rabbits several days later, one of 4.05 units per cubic centimetre after incubation. Similarly a solution of p_H 8.6 had potencies of 5.15 and 4.65 units per cubic centimetre respectively. This finding is of importance for it indicates that in the presence of mild alkali a change may occur without any concomitant appreciable change of physiological activity. The nature of such a change is extremely problematical.

Incubation of insulin-glucose mixtures for 24 hours at 37° at acid reactions (p_H 3-4) is not attended by any apparent effect on the surface charge of the

protein. This finding is in agreement with the fact that insulin retains its physiological activity in acid solution to a greater extent than in alkaline solution. The changes in reaction which occur on incubation are also indicative on this point; the following values were noted for insulin-glucose mixtures.

Original p_H .		p_H after incubation.
3.0	3.07
3.84	3.90
8.64	8.54
9.45	8.08

The authors are indebted to the Government Grant Committee of the Royal Society for a grant with which the insulin used in the above research was purchased, and to Messrs. Boots Pure Drug Co., Ltd., Nottingham, for supplying fully tested insulin of high purity at a generous price.

Summary.

(1) The electrophoresis of insulin alone and in presence of glucose has been determined over a range of p_H 2.7–9.6, gold sol fully protected by the protein being utilised as an indicator of the migration.

(2) Under such conditions insulin exhibits an isoelectric point of approximately p_H 5.4 and an isoelectric zone or region of insolubility of p_H 4.5–6.5.

(3) No evidence of a glucose-insulin reaction *in vitro* either at room temperatures or following incubation at 37° for 24 hours and at the reaction of the blood (*i.e.*, p_H 7.4) is forthcoming from the data obtained.

(4) At reactions of greater alkalinity than that of the blood, namely, p_H 8.9–5, a reaction between glucose and insulin is indicated and is emphasised by incubation at 37°.

(5) Incubation at 37° of insulin at alkaline reactions is followed by a change in the electrokinetic potential of the protein micella. This change is not accompanied by the loss of physiological potency.

(6) Insulin behaves electrophoretically as a chemical entity, *i.e.*, the separation into an active and an inactive fraction under the influence of the applied electromotive force could not be detected.

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*The Isoelectric Point of Serum-Globulin as Determined by
Kataphoresis.*

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PRIDEAUX.

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Following the determination of the isoelectric point of insulin by kataphoretic methods (Howitt and Prideaux, 1932) it appeared to the authors that a similar determination for serum-globulin would be of interest owing to the globulin-like character of insulin. A comparison between the kataphoretic behaviours

of these two proteins which are physiologically so different would also be afforded.

Previous determinations of the isoelectric point of serum-globulin have generally been based on methods of maximum insolubility, or coagulation. Thus Kugelmass (1922) obtained a value of p_H 4.55, whilst Krebs (1925), employing the coagulative action of proteins on a gold sol, found p_H 5.4. The experiments of Rona and Michaelis (1910, 1927) gave the value 5.44 in close agreement with that of Krebs. Reiner (1927), however, has stated that serum-globulin has a broad isoelectric zone and is not a homogeneous substance. The findings of this worker are probably explained by the conclusions of Svedberg and Sjögren (1928) that serum-globulin is a chemical entity, which degraded into fractions during normal treatment, such as "salting-out" with ammonium sulphate.

Experimental.

Serum-Globulin.—Ox-serum, free from hæmoglobin, was treated according to the method of Svedberg and Sjögren (1928). The serum was half-saturated with ammonium sulphate, the precipitate filtered off, washed with half-saturated ammonium sulphate solution and dissolved in 10 per cent. sodium chloride solution. The globulin was re-precipitated with ammonium sulphate, and washed, and again dissolved in 10 per cent. sodium chloride solution. This was repeated a second time and the washing continued until the filtrate did not respond to the biuret test. The final residue was dissolved in a 0.2 M solution of the buffer used in the experiments and dialysed against a similar solution which was frequently changed until the outer liquid was free from chloride and sulphate even after a period of 48 hours. The protein content of the solution was determined by drying to constant weight at 100°, allowance being made for the weight due to the constituents of the buffer. By dilution with M/5 buffer a stock solution containing 0.2 per cent. globulin in M/5 buffer was obtained.

The technique of the kataphoresis experiments was exactly parallel with that used in the case of insulin. Thus to 10 c.c. of the stock globulin solution were added 10 c.c. of the stock gold sol followed by the requisite amount of acid or alkali, and the solution made up to 50 c.c. This gave a final concentration of 0.02 per cent. in serum-globulin, 0.01 per cent. in gold and 0.02 M in buffer. Solutions of corresponding reactions for the "buffer column" were prepared as before.

The migration velocities obtained are given in Table I and in graph form in fig. 1, where that of insulin is also shown for comparison.

Table I.—The Electrophoretic Velocities of Serum-Globulin.

pH .	Temperature.	W_1 .	W_2 .	E .	$v = \frac{(w_1 h_1 + w_2 h_2) 1000}{3600 \times E} \mu/sec.$	$v = \eta/\eta_{10}$.
	°	cm./hr.	cm./hr.	volts.		
2.85	18.5	3.7	3.2	154	+1.66	+1.64
3.38	18	3.8	2.8	156	+1.51	+1.51
3.90	18	3.8	2.4	160	+1.34	+1.34
4.25	17.5	2.6	1.1	160	+0.75	+0.76
6.90	15	4.0	1.5	174	-0.98	-1.06
7.63	18	3.6	1.9	155	-1.18	-1.18
8.75	18	2.6	2.0	159	-1.04	-1.04
9.58	18	3.8	2.6	159	-1.45	-1.45

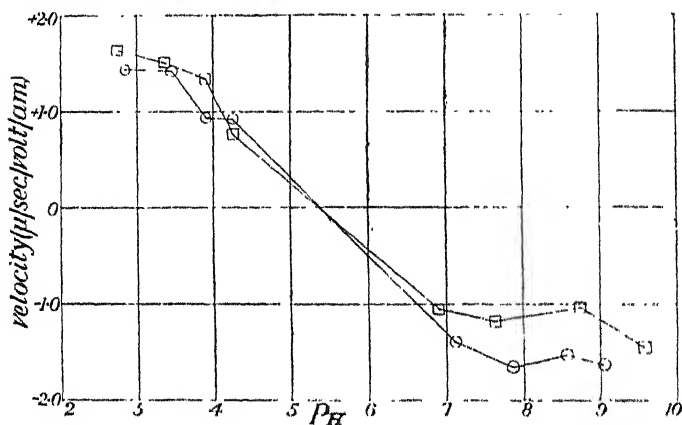


FIG. 1.

○ Insulin. □ Serum-globulin.

Discussion.

The curve for the electrophoretic velocities of ox-serum-globulin is of a type similar to that of insulin. At a reaction of p_H 5.35 the curve cuts the line of zero velocity. The globulin exhibits a zone of insolubility closely approximating to that of insulin and this comparatively wide region of insolubility influences the accuracy of the determination of the isoelectric point by katalaphoresis.

The value of p_H 5.4, however, may be accepted as a fairly close approximation to the true value.

The migration velocities on the alkaline side are of a lower value than the corresponding velocities for insulin, whilst on the acid side there is a tendency for the values to be greater than those of insulin.

Summary.

The electrophoresis of ox-serum-globulin in presence of a gold sol has been determined over a range of p_{H} 2·7–9·6.

Under such conditions the globulin exhibits an isoelectric point of approximately p_{H} 5·4 and a zone of insolubility of p_{H} 4·5–6·5.

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Certain Pathological Effects of Ultra-Violet Radiation on Mosquito Larvæ and Pupæ.

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[PLATE I.]

In the course of recent work on the possible effect the ultra-violet wave-band may have on the activation of ovarian function in female mosquitoes, it became apparent that mosquito larvæ are highly susceptible to a remarkable form of injury by radiations from the unshielded mercury-arc generated by the ordinary Cooper-Hewitt vacuum type of quartz mercury-vapour lamp.

This effect upon the larvæ has been studied, and an attempt was made (1) to determine the wave-length of the radiations responsible for the injury by means of screens interposed between the lamp and the larvæ; and (2) to ascertain the physiological and histological nature of the injury.

So little reliable information was readily obtainable from available sources on the physics of the ultra-violet radiations, that I wrote to Sir Leonard Hill, F.R.S., for advice in this matter and he kindly introduced me to his colleague Dr. H. J. Taylor, of the Research Department of the London Light Clinic. It was arranged between Dr. Taylor and myself that preliminary observations should be made on the consequences of irradiating larvæ under particular

screening materials, and that the results should be surveyed from the physical standpoint.

The actual wave-length ranges of my particular screening materials have been accurately determined subsequently by Dr. Taylor. To him, I am greatly indebted also for much information in regard to the physical characteristics of the radiations produced by the mercury-arc, and for the ultra-violet spectrograph plate.

A.—*Preliminary General Observations. U.-V. Irradiation of the Larvæ of Aedes (Stegomyia) ægypti, L.*

On exposure to the unshielded radiation from the quartz mercury-arc lamp, the larvæ within 45 seconds exhibit an apparent irritation along their abdominal segments and over the siphon. This is evidenced by the larvæ bending their bodies and applying their mouth-brushes vigorously over the affected areas. If the larvæ are exposed in an open watch glass at 11½ inches from the unshielded lower surface of the arc tube (fitted with a parabolic reflector) operating on a D.C. current of 4.5 amperes at 100 volts, little or no noticeable injury is immediately apparent. If any change occurs immediately after irradiation, it is a tendency on the part of the larvæ to hang at the surface of the water somewhat abnormally; that is, with a slight ventral curvature of the anterior part of the body. The larvæ are, however, quite active and capable of performing all the normal swimming movements, but gradually a condition of partial paralysis ensues, then they hang from the water surface with a conspicuous ventral curvature and their swimming movements become clumsy and quite abnormal. This condition can generally be observed in some degree within an hour after irradiation, is usually quite conspicuous within 6 hours, and very pronounced within 24 hours. In this state the growth of the larvæ ceases, feeding is practically stopped, they become feebler and die at the end of a variable period of from 3 to 7 days. Before death takes place the larvæ sink to the bottom of the water, and there make the same writhing movements as occur in the case of larvæ poisoned and asphyxiated by oil.

B.—*Effect of Irradiation of 3 minutes' duration under various shields.*

(1) *With ⅜-inch "Vita" glass sheet interposed between the lamp and the larvæ in a watch-glass.*—Larvæ exhibit irritation within 45 seconds, but at the end of 24 hours are quite normal.

(2) *With two folds of "membrane" cellophane interposed.*—Result similar.

(3) *With ⅜-inch sheet of window-glass interposed.*—Result similar.

(4) *With $\frac{1}{2}$ -inch thickness of window-glass interposed.*—Complete absence of irritation during 3-minute period of irradiation, and even after multiple exposures of 3 minutes' duration. No paralysis.

Indications.—Since with the use of these shields the paralysis is not induced, whereas the irritation occurs in all except No. 4, it seemed clear that the irritation and the induction of paralysis were not correlated. It was thought possible that the cause of the paralysis lay in the visible or infra-red spectrum, but if so, it seemed a little difficult to understand why Cellophane of "membrane" thickness should be sufficient to prevent the injury to the larvæ which occurs when there was nothing interposed between them and the mercury-arc. The paralysis effect also takes place when quartz sheet is interposed.

After consideration of these observations Dr. H. J. Taylor indicated upon what lines the analysis and physical explanation should follow, and on receipt of the spectrograph, Plate 1, Table I was drawn up, together with the interpretation of the theoretical analysis.

Interpretation of the Theoretical Analysis, Table I.

1. *Irritation within 45 seconds, but no paralysis 24 hours later, using "Vita" glass shield.*—Paralysis would appear to be due to wave-lengths below "U," and irritation, due either to ultra-violet between "U"—4000 Å.; possibly to part of the visible spectrum; or to the injurious infra-red range between 8000–13000 Å.

2. *Irritation but no paralysis with Cellophane.*—Paralysis would appear to be due to wave-lengths below "V," and irritation, similarly, to ultra-violet between "V"—4000 Å.; to the visible, or to the infra-red range—as in No. 1.

3. *Irritation but no paralysis with $\frac{1}{2}$ -inch glass.*—Again, paralysis would appear to be due to wave-lengths below "X," and irritation to ultra-violet between "X"—4000 Å.; to the visible spectrum, or to the infra-red—as in No. 1.

4. *No irritation and no paralysis with $\frac{1}{2}$ -inch glass.*—As the $\frac{1}{2}$ -inch glass transmits both visible and infra-red radiations, but ultra-violet only above "Z"; therefore, paralysis would seem to be due to ultra-violet radiations somewhere between 2150 and "V" Å., and irritation clearly is not due to either the visible or to the infra-red spectrum, but to the ultra-violet somewhere between 2150 and "X" Å.

The spectrograph, Plate 1, made by Dr. Taylor, shows the exact transparency range of the particular shields that were employed.

Dr. Taylor subsequently pointed out to me that in order to determine the upper and lower limits of the wave-lengths concerned in the production of the

Table I.—Physical Analysis of the Effect of the U.-V. Irradiation in accordance with the Observations made by M.E.M.

		Complete Spectrum of Mercury-arc.							
X-rays	a	Limits of Quartz Mercury-Arc Spectrum (spectrograph).						//	
		1300	2150	U.-V.	4000	Visible	8000	Infrared	200,000 A.
		8000 - 13,000							
U.-V. Below Mercury-Arc Spectrum. A trace of radiation down to 2100 A.		"U"		"Vita" glass		//		(55,500 A.)	
		"V"		"Cellophane"		//			
		"X"		1/8-inch glass		//			
				"Z" 1/2-inch glass					

Note.—The upper limit of the quartz mercury-arc, here shown, is true only when the lamp has reached its maximum temperature, when the heated quartz itself then acts as an emitter of long infra-red rays. When the lamp is relatively cold after it is first started the upper limit of the quartz is about 45,000 A.

paralysis and irritation effects separately, a series of exposures of increasing duration should be given under the different shields. This is on account of the fact that the transition from transparency to opacity to the radiations of different wave-length on the part of the shields is not abrupt, but is exhibited as a more or less rapid transition associated with a proportional decline in the intensity of the radiation which is being cut off.

Proof that the Condition of "Paralysis" is induced by Radiations between 2150 A. and a little above 2600 A.—As I have shown in the results of the preceding experiments, the larvæ which were exposed under two folds of Cellophane for a period of 3 minutes exhibit no subsequent paralysis at the end of 24 hours. It was found, nevertheless, that signs of paralysis in these larvæ were present at the end of a further period of 24 hours, obviously, therefore, the larvæ irradiated with rays of wave-lengths not lower than 2220 A. must have been subject, in a minor degree, to paralysis-inducing radiations, and that their wave-length range must extend somewhat above 2220 A. Separate

batches of larvæ were accordingly subjected under "Vita" glass to exposures of a duration of 6 minutes and multiples thereof up to 72 minutes under exactly the same conditions in regard to the mercury-arc as in the previous experiments. Ninety-six hours later most of the larvæ in all the tubes below the 54 minutes irradiation (three separate series, 24 tubes in all) had pupated and were quite healthy, while the tubes of the 54, 60 and 72 minutes exposures held larvæ that were "injured"? "certainly injured" and "conspicuously injured" respectively. In the tubes marked "injured"? one of the larvæ succeeded in pupating and emerging as a female of normal appearance. The remainder of the larvæ in this and other "injured" (?) tubes either died before or soon after pupation. In the "conspicuously injured" tubes, all died as larvæ.

It was thus established that the paralysis-inducing radiations in attenuated intensity (or potency) were present under "Vita" glass in a degree sufficient to produce pathological effects upon the larvæ after an exposure to irradiation of not less than 54 minutes. Consequently, since the lowest limit of transparency to ultra-violet rays in my shield has been found to be approximately 2600 Å., it was disclosed that the "active" radiations must extend slightly beyond 2600 Å. To settle the question of the approximate extent to which the "active" radiations exceeded 2600 Å., other batches of larvæ were irradiated under the $\frac{1}{8}$ -inch window glass for periods of 6 minutes and multiples thereof up to 72 minutes, and one batch for 144 minutes. Ninety-six hours later all the larvæ in each of the 39 tubes (3 series in all) had either pupated or still existed as uninjured larvæ which later completed their metamorphosis in normal manner.

It was thus shown that the "active" radiations did not extend as high as 3140 Å. A closer estimation of the wave-length range is being made by means of quartz refractive apparatus.

The Minimum Exposure required for the Production of "Paralysis."—When using a quartz mercury-vapour lamp which had been in operation for not more than 150 hours it has been shown by serial exposures of larvæ for various periods that the induction of subsequent "paralysis" occurs by an exposure of approximately 45 seconds to the unshielded lamp when the lower surface of the burner is $11\frac{1}{2}$ inches from the watch-glass holding the larvæ. "Paralysis" is then generally noticeable within a period of 48 hours after such irradiation.

The Relative Susceptibility of Larvæ at various Instars.—The bodies of larvæ of *Aedes (Stegomyia) aegypti*, L., in the earliest stages of development are nearly

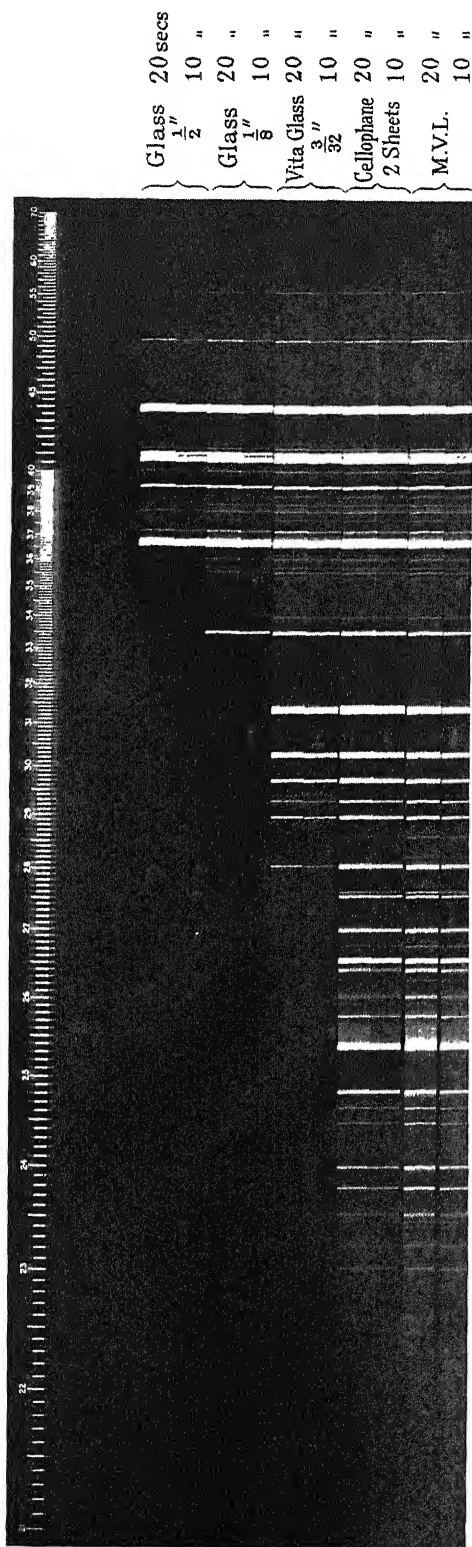
transparent to visible light. As they become older this transparency lessens until, when they reach the late 4th instar, the body is almost opaque, and generally contains numerous pigment granules within the fat-body. Experiments were carried out to determine the relative susceptibility of the larvæ at different ages to injury by ultra-violet radiations. The results from one of a uniform series of experiments, which gave comparable indications, are shown in Table II.

Table II.—Larvæ Irradiated for 3 Minutes in Open Watch-glasses at 12 inches from the Mercury-arc.

Date.	Number of larvæ.	Age.	After irradiation.	20 hours later.	End result.
19th	12	1st-2nd instar	Slight curvature	8 normal (?) 4 badly injured	10 dead, 2 badly injured on 21st. All dead 22nd.
19th	12	2nd-3rd instar	Slight curvature	12 alive; dying, badly injured	All dead by 21st.
19th	12	3rd-4th instar	Slight curvature	4 dead, 8 badly injured	8 dead, 4 badly injured on 21st. All dead by 22nd.

It is evident from Table II, and by the comparable results obtained in experiments of the same kind, that the larvæ at all stages of development are more or less equally susceptible to injury and death from exposure to the radiation. The ventral curvature in the young larvæ was, however, much more marked than in those at later stages of development, and in many cases the young larvæ surviving at the twentieth hour were curved to such an extent that they exhibited an actual comma-contour.

The Susceptibility of Pupæ.—On the whole, pupæ have proved considerably less susceptible to injury than larvæ. Immediately after pupation the insects seem almost as easily damaged as 4th instar larvæ, but as the puparium darkens the insects become to some extent increasingly resistant. The majority of pupæ, however, if collected indiscriminately from jars containing numbers at various ages, when subjected to a 6-minute exposure are severely injured and die before the emergence of the adult. Large numbers reach the initial stages of ecdysis only to die during the process of the rupture of the dorsal suture. Enfeeblement, due to severe tissue injury, has been found to be the cause.



“*Irritation*” and “*Paralysis*.”—As I at first surmised, the irritation and paralysis effects do not seem to be correlated. Larvæ can be subjected to the irritating radiations by the interposition of a shield of window-glass between the larvæ and the lamp for long periods without ill-effect. Larvæ of *Aedes* (*Stegomyia*) *egypti*, L., exhibit the irritation by bending and applying their mouth-brushes vigorously over the affected areas. This action is maintained during continuous irradiation under window-glass sometimes for as long as 10 minutes, but usually after 5 minutes the larvæ become tolerant or cease to feel the irritation. In the case of larvæ of *Culex* (*Culex*) *pipiens*, L., the reaction is different. No application of the mouth-brushes occurs, although the larvæ are evidently experiencing great irritation. They are highly agitated, swimming at an increased rate to every part of the watch-glass in an effort to escape the radiations, but after a time they, too, become tolerant and rest quietly at the surface under the full glare of light, feeding normally. “Negative phototropism,” in all probability, is nothing more or less than the ordinary phenomenon of fear, or the evasion of an abnormal or possibly dangerous environment under the guidance of intelligent perception, until experience teaches that the anomalous conditions are apparently not disastrous to the organism.

The Histological Nature of the Injury.—During the preceding experiments it was soon recognised that what I have here hitherto termed “paralysis” was actually symptomatic of an injury of an unusual kind. Primarily I had realised the need for close examination of the separate tissues to discover which specifically were being acted upon by the ultra-violet radiations, but such peculiar changes—in the nature of histolysis—were soon observed under the binocular microscope, or even by means of a simple hand-lens, in the internal tissues and morphology of the irradiated larvæ, that the type of histological study first contemplated was radically altered. It was found that the rate of the induction of these tissue changes was directly proportional to the duration of irradiation over fairly wide periods, and, moreover, that the death of the larvæ as surely followed irradiation of short duration as it did of long, the only difference being that the progress of the process of tissue change and the ultimate death of the larvæ was inversely proportional to the duration of the irradiation. Nevertheless, it was found that death in both cases followed the final establishment of a comparable condition in regard to the pathology. Hence it is evident that the tissue histolysis, once started, is progressive: the rate of histolysis being correlated with the duration of exposure.

To show the course of events it will be sufficient to quote the records of one of a comparable series of my experiments.

Twelve 3rd-4th instar larvæ in open watch-glasses were irradiated for 6 minutes. Slight partial paralysis was immediately apparent at the end of the exposure, as evidenced by ventral curvature. Examination under the binocular microscope revealed, however, no perceptible tissue change. The larvæ were then set aside for a period of 20 hours in an incubator at a temperature of 30° C. On re-examination remarkable changes in the tissues were at once apparent. Over practically the whole of the dorsal area of the thorax and abdomen detachment and dissolution of the connective tissues from the under-surface of the integument was well advanced. Disintegration and dissolution of the annular segmental masses of the fat-body was in progress in such a way that filaments and strands of the fat-body were loose and were waving about within the segments. A clear space (ultimately found to be filled with transparent fluid) was being formed between the dorsal inner wall of the integument and the disintegrating tissue surfaces. Partial paralysis of the respiratory motor mechanism was marked. The pulse rate and tone of the heart were distinctly lowered; its peristaltic-wave no longer travelled from between the 7th and 8th segments of the abdomen with normal impulse, diverging the longitudinal tracheæ over their entire length, but instead travelled only a short distance anteriorly, causing merely a feeble and limited divergence of the tracheæ. The longitudinal tracheæ were partially (sometimes completely) collapsed due to paralysis of their dilator-compressor nerve-muscle mechanism. At all parts of the body the stream of the cœlomic fluid in a posterior direction towards the ostia could be seen to be carrying the remnants of the dissolving tissue elements. (This phase is particularly well marked in the larvæ of *Culex* (*Culex*) *pipiens*, L.: less so in *Aedes* (*Stegomyia*) *egypti*, L.)

The larvæ found great difficulty in performing their swimming movements, owing partly to the paralysis, and partly to the dissolution of the abdominal muscles, consequently, the insects could only make indecisive locomotive movements. In some cases paralysis also affected the muscles operating the labellæ of the siphon, though more often this was not so, and the siphon itself functioned perfectly. The action of the mouth-brushes was in some cases severely injured, while in others they could be operated vigorously though in an abnormal manner, in that the hairs of the brushes were not individually extended in the usual way with the result that they were no longer so effective. Nevertheless, on the whole, the motor mechanism of the mouth-parts, antennæ and siphon were more under control than the rest of the motor system. Occasionally, the suspensory ligaments of the alimentary canal were so far

destroyed as to permit the protrusion of the greater part of the intestine and malpighian tubules from the anus.

After these observations had been made the larvæ were re-examined at 3-hourly periods up to the twenty-ninth hour after irradiation, and then again at the forty-fourth hour. The progress of the condition of dissolution of the tissues was found to be continuous, and rapidly involved the soft tissues, finally leaving unaltered only the tracheal system, and chitinous integument. By the forty-fourth hour most of the larvæ were already dead, and all had died by the fiftieth hour. Shortly before death the insects were so injured and enfeebled that they were unable to maintain their equilibrium in the water or their breathing position at the surface, and sank to the bottom where they continued slight movement for some time. Immediately before, or after death, the bodies presented a most striking appearance, there being merely a ribbon of intact tissue extending along the ventral aspect of the body-cavity. The area above this ribbon was quite transparent but for the ramifications of the tracheal system, yet by placing the larvæ in boiling water or other fixative the area was rendered opaque, thus proving that this region was occupied by a coagulable fluid—the product of the complete or partial histolysis of a number of different tissues. Muscles, nerves, fat-body (including the contained pigment), intestine, malpighian tubules, etc., had histolysed, partly or entirely, to form, under the action of electro-magnetic radiations of particular quanta, a homogeneous transparent fluid.

This is the macroscopical picture to be observed in larvæ after such irradiation. The microscopical aspect is not less striking, but considerably more difficult to analyse. Several months of further study will be necessary to make out all the details, but the most conspicuous points so far noted in dissected larvæ and microscopical sections are as follows:—

- (1) The close adherence of the fat-body tissue to the under surface of the integument is broken. The disintegration of the parietal layer on the dorsal surface which has received the direct impact of the radiations is the first part to show signs of dissolution. The protoplasmic boundaries of the vacuoles containing fat enlarge, due apparently to the hydrolysis of the fat itself. Lacunæ appear between the cells; the hydrolysed fat is liberated and disintegration of the cell structure follows.
- (2) The respiratory motor mechanism is, in some cases partially, and in others completely, paralysed, as indicated by the collapse and dilation of the longitudinal tracheæ and their chambers.

- (3) In severely injured larvæ the intestine dilates and becomes more or less hyaline. Histolysis of the intestinal tissue, however, only rarely occurs before the death of the larvæ.
- (4) In general it appears that histolysis of the cells of all the different tissues is preceded by evidence of injury to the nucleus. The nucleus enlarges and degenerates while the protoplasmic contents become hyaline and finally shrink, apparently under a process of liquefaction.
- (5) Great as is the injurious effect upon all the soft tissues, it seems clear that the most profound changes occur in the tissues of the fat-body.

Experiments to ascertain the effect of Local Irradiation. Several methods were tried of exposing only a part of the larval body to the action of the rays, in such a way that the energy intensity of the radiation was not altered by the interposition of any material other than water in the watch-glass. Great difficulty was experienced in holding the active larvæ immobile without causing mechanical injury, until the following method was evolved.

A microscopical glass slide is cut into pieces 1 inch square, and two glass strands, drawn from glass tubing, are cemented 2 mm. apart with dental wax to the glass square, one on each side of the median line. The diameter of the two glass strands should be about 1 mm. The space enclosed by these two strands is then filled with dental wax, and after the solidification of the wax it is smoothed off parallel with the upper arcs of the strands by means of a hot needle. When the wax is quite cold a small chamber about 15 mm. in length is excavated in the wax between the glass strands over the middle area of the glass square with the aid of a cold dissecting-needle. The glass bottom of this chamber is carefully cleaned so that no wax remains adherent to the glass surface. The small chamber thus formed is then filled with water, and a 3rd or 4th instar larva is placed therein. Surplus water from the cell is drawn off by capillary pipette, and the space accommodates the larvæ in such a way that little or no lateral movement is possible. A piece of tin-foil is now folded to form three overlapping sheets (a precaution necessary since the tin-foil often bears "pin-holes") and the folded sheet cut to a size of about 20×10 mm. The sheet of foil is then pierced near its centre with a No. 8 sewing needle, the "burr" on the under side is flattened by pressure on a glass surface, and the hole made almost circular by repassage of the needle. The foil is laid carefully over the upper surface of the chamber, and adjusted until one of the abdominal segments or part of the thorax of the larva is seen centrally situated

through the hole. Cement one lateral free edge of the foil to the glass square by touching the glass and foil edge at the line of contact with a hot needle carrying a drop of melted wax. Gentle pressure is then applied to the foil over the chamber and over the whole length of the glass strands. This action partly secures the larva in the required fixed position. The opposite lateral edges of the foil are later fixed to the glass square in a similar manner. The cell may now be turned over and the larva in its chamber seen from underneath. Finally, to secure the larva rigidly, the foil is indented gently with a blunt needle point just in front and just behind the head and tail respectively, taking care, of course, not to rupture the metal. Replace the cell, foil-side uppermost, on the stage of the binocular microscope and with a capillary pipette deposit drops of water over the hole in the foil until no more water is received by the chamber. The cell may then be submerged in a small petri-dish containing a shallow depth of water, for the local irradiation of the larva. After the required period of exposure the larva may be released into a vessel of water containing a food supply, by simply lifting one lateral edge of the foil while the cell is held below the water surface.

Experiments conducted on the effect of local irradiation with cells of this kind have been most satisfactory. The result of such experiments has been to show that the tissue injury is at first limited to the area exposed by the hole in the foil, but that it rapidly becomes extensive, and spreads finally all over the body within a few hours after irradiation. The progress of the tissue dissolution is, however, considerably slower compared with larvæ which have received a simultaneous irradiation of all parts of the body.

Obviously, the significance of the results of this series of experiments is of supreme importance in providing a possible clue to the cause and process of the tissue changes. In the first instance the cells of the area exposed to the radiations through the hole in the foil must have been directly injured by the action of the rays, but subsequently tissues which have been completely protected from direct irradiation are similarly affected. Lewis's work (1927) and his theory of the liberation of H— substance is, in this connection, of special interest.

Notwithstanding the serious nature of the tissue damage that results from irradiation, sub-lethal dosage, while causing conspicuous injury to certain larvæ, may not prevent normal growth and metamorphosis in others. Moreover, the imagines derived from such slightly injured larvæ are morphologically, anatomically and sexually normal.

Summary.

(1) Irradiation of the larvæ and pupæ of *Aedes (Stegomyia) aegypti* L. and *Culex (Culex) pipiens* L. with ultra-violet radiation has been shown to cause a peculiar form of fatal injury to the insects.

(2) Particulars are given of the technique employed, and of the results of numerous experiments that were carried out to determine the wave-lengths of the radiations responsible for this particular type of injury, the minimum exposure required for the production of "paralysis," to ascertain the relative susceptibility of larvæ of various instars, and also of pupæ, and the effect of "local" irradiation.

(3) The histolysis of the tissues which results is found to be of a progressive type, affecting not only the cells which have themselves been subjected to irradiation, but also the adjacent cells from which the injury continues to spread.

(4) The histological nature of the injury is briefly described.

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Factors affecting the Breeding of the Field Mouse (Microtus agrestis).
Part II.—Temperature and Food.

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Introduction.—In Part I (1931) we described experiments on the effects of light on reproduction in the field mouse. The conditions under which the control and experimental mice were kept was described in full. The control mice were kept for a year under summer conditions of temperature, food and daily period of exposure to light (15 hours), while the experimental mice had the same conditions of temperature and food, but light for only 9 hours daily. We call this the “winter light” experiment. The control mice bred throughout the year, but the winter-light mice scarcely at all.

Concurrently with the winter light experiment, two other experiments were carried out against the same control. These were the winter temperature experiment and the winter food experiment, which are described in this paper.

Winter Temperature Experiment.—Except as regards temperature, the experimental mice were subjected to exactly the same conditions as the control mice, namely, summer food (growing grass or the equivalent) and 15 hours of electric light each day, from a 60-watt bulb suspended about 3 feet above the cages. The construction of the four cages, and the number of mice of each sex in each, were as nearly as possible the same as in the controls. One male was kept in each cage. The number of females under winter temperature conditions was on the average 8·4, as against 7·5 in the controls. The acclimatisation of the mice and their periodical removal and renewal was described in the earlier paper. Thirty-six females passed through the winter temperature cages during the months of the experiment.

The only difference from the control conditions was that the mice were kept at a low temperature. In the cold part of the year they were kept in an unheated wooden house with the windows open. In the warm part they were kept in a special ice-box, designed and constructed by Mr. S. J. Baker (see

fig. 1). The ice-box consisted of two boxes of insulating board, one box fitting inside the other. The space between the two, which was everywhere 5 inches across, was filled with hay, except that the bottom was filled up with layers of insulating board so as to give sufficient strength for a man to enter the smaller box to feed the mice and clean out the cages. The inner box was divided into two compartments, one containing the cages, and the other

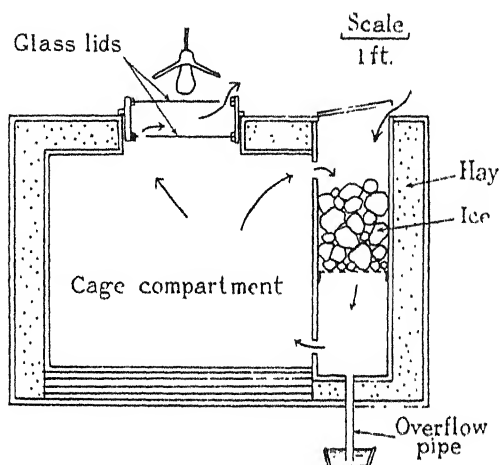


FIG. 1.—Ice-box for winter temperature mice. The arrows indicate the circulation of air.

lined with sheet iron, containing ice. The latter compartment had a sump with a pipe to conduct water away. The lower end of the pipe dipped below a basin of water, to prevent the upward entry of warm air into the ice-box. The cage-compartment was connected with the ice-compartment by apertures at the top and near the bottom, to allow the free circulation of air. Both the cage-compartment and the ice-compartment opened on the top, the aperture of the former being large enough to admit a man. The lid of the ice-compartment was simple, but the lid of the cage-compartment was complex. It consisted of a flat box with a glass top and glass floor, about 5 inches apart. The 60-watt electric bulb was suspended immediately above the upper glass. The air between the two glasses served for insulation. Fresh air entered the ice-compartment by a space under its lid. On being chilled and sinking to the bottom, it passed through the lower aperture into the cage-compartment. On being warmed by the mice, part of it returned to the ice-compartment by the upper aperture, and part was drawn out through special spaces provided in the glass lids. This arrangement ensured the circulation of a sufficiency of fresh air.

During the months of May, June, July and August we were unfortunately unable to keep the ice-box sufficiently cold, and therefore we shall only consider the remaining months of the year. Fig. 2 shows the temperatures maintained during these months. The control temperatures are also shown.

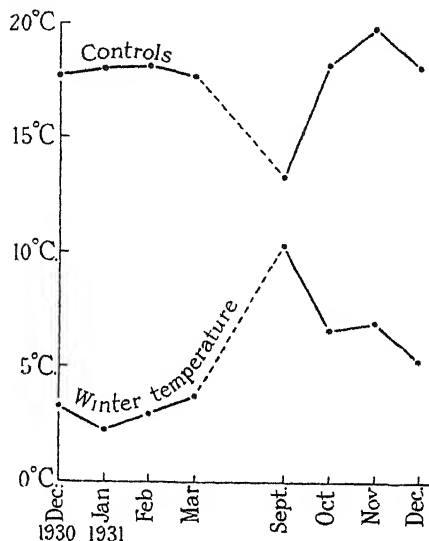


FIG. 2.

The breeding results are given in Table I. September was regarded as a month of acclimatisation to cold, and the births which occurred in this month

Table I.

Date of birth.	Controls.	Winter temperature experiment.
1931.		
January 20	4	—
February 18	1	—
March 4	0	—
March 14	4	—
March 18	3	—
March 24	4	—
April 16	3	—
October 6	4	—
October 8	3	—
October 27	4	—
November 5	2	—
November 7	—	4
November 9	0	—
November 13	—	4
November 19	—	4
November 19	—	0
December 7	0	—
December 14	6	—
December 21	0	—
	38	12

are not recorded. The numbers are the numbers of young in each litter. The symbol "0" indicates a pregnancy followed by the absorption of the young.

It appears certain that lowered temperature hinders reproduction. It might be thought that the results were due to the unfavourable conditions in the ice-box, apart from the temperature. There was obviously less fresh air, and the humidity was slightly greater (87 instead of 84) (see Appendix). However, these factors cannot have affected the reproduction, because the mice in the ice-box bred rapidly in the warm months, when we were unable to keep the temperature low. During these months they actually produced many more young than the controls.

The males were removed and killed every 3 months, to find whether their reproductive organs were affected by the lowered temperature. New acclimatised males were substituted. The result of the examination of the males is shown in Table II. The control males killed every 3 months throughout the year of the experiment are also shown. The table explains itself, except the fifth column. This shows the activity of the sperms in a buffered glucose-saline fluid at 37° C. The activity is recorded according to a scheme in which the

Table II.

Weight of mouse (grams).	Weight of testes (milligrams).	Weight of vesiculae seminales (milligrams).	Abundance of sperms.	Activity of sperms.	Fecundity.
Controls.					
43	560	110	Abundant	3	Fecund.
33	320	200	"	2	Fecund ?
28	350	160	"	3	Fecund.
34	550	90	"	3	Fecund ?
30	270	70	"	3	"
33	420	200	"	3	Fecund.
31	450	110	"	3	"
30	250	100	"	3	"
25	100	10	Few	0	Non-fecund.
Winter Temperature.					
33	500	180	Abundant	3	Fecund.
31	360	100	"	3	"
37	430	120	"	3	"
32	390	70	"	3	"
32	280	120	"	3	"
33	450	160	"	3	"
27	480	100	"	3	"
17	180	20	"	3	Non-fecund ?
28	190	30	"	2	Non-fecund.

maximum is 3+, no activity whatever is 0, and 3, 2+, 2, 1+ and 1 are intermediate degrees. The fecundity (last column) is judged from the second third, fourth and fifth columns.

For comparison, it must be mentioned that wild adult male *Microtus* in winter have testes of 10 mgm. or less, vesiculæ considerably smaller still, and no sperms at all.

The table shows that there was no significant difference between the fecundity of the control and winter-temperature males. We must conclude that the effect of lowered temperature in checking reproduction is an effect upon the females. As was explained in our first paper, shortage of females prevented us from killing them off at quarterly intervals for microscopical examination of the ovaries, and at the conclusion of the experiment every female was required for building up a stock for further experiments. We therefore do not yet know how the lowered temperature renders the females less fecund.

Parkes and Brambell (1928) found that the house-mouse has regular oestrous cycles if kept at about 0° C. with abundance of food. Pregnancies occur if copulation is allowed.

Winter Food Experiment.—Except as regards food, the experimental mice were subjected to exactly the same conditions as the controls, namely, 15 hours of electric light a day and summer temperature. The four cages were exactly similar. Each contained one male. The number of females under winter food conditions was on the average 6.0, as against 7.5 in the controls. The acclimatisation of the mice and their periodical removal and renewal was described in our earlier paper. Altogether 51 females passed through the winter food cages.

Before starting the experiment we tried to find out, from the literature and from observations, what *Microtus* eats during the winter. Unfortunately we were unable to ascertain this with certainty, but it appears probable that it eats grass (non-growing, of course) and stores of grass-seed which it has prepared before. We therefore fed our winter-food experimental mice on hay, rye-grass seed, and a very small amount of growing grass. Each mouse ate about 3 gm. of hay and only 1½ gm. of growing grass each day, and an unlimited supply of grass seed was provided. The controls ate about 30 gm. of growing grass a day, no hay, and no grass seed. The food of the control mice was discussed in our earlier paper.

The breeding results are given in Table III. The numbers indicate the number of young born, the date being the date of birth. The sign "0" indicates a pregnancy resulting in the birth of no young, owing to absorption.

Table III.

Date.	Controls.	Winter-food.
1931.		
January 20	4	-
February 13	—	4
February 24	—	3
March 4	0	3
March 10	-	4
March 12	-	3
March 12	-	5
March 14	4	-
March 18	3	-
March 24	4	-
April 11	-	3
April 15	-	3
April 16	3	2
May 9	-	0
May 10	-	4
May 11	0	-
May 18	0	-
May 18	0	-
May 18	0	-
May 21	-	4
May 22	-	2
June 6	-	4
June 15	-	4
June 19	—	4
June 28	-	4
June 29	-	0
July 9	-	0
July 10	-	6
July 15	2	-
July 28	-	4
August 4	-	6
August 5	0	-
August 5	0	-
August 6	-	0
August 11	—	3
August 14	0	0
August 23	—	4
September 18	4	—
September 21	—	3
September 22	3	—
September 28	—	4
October 6	4	—
October 8	3	-
October 15	—	2
October 21	—	1
October 24	—	2
October 27	4	—
October 30	—	0
November 5	2	—
November 9	0	0
November 12	—	1
December 7	0	5

Table III—(continued).

Date.	Controls.	Winter-food.
1931.		
December 7	—	0
December 8	—	1
December 14	6	—
December 19	—	4
December 21	0	—
December 22	—	3
1932.		
January 6	—	4
January 9	—	5
January 11	—	3
Totals	47	117

It will be observed that the winter-food mice had significantly more young than the controls. It is evident that a large supply of growing grass is not necessary for rapid reproduction in *Microtus*, although the natural breeding season corresponds roughly with the period when the growth of grass is most rapid. The grass seed provided may have contained vitamin E, though it was provided perfectly dry and never germinated.

The fecundity of the male winter-food mice was judged by our standard criteria, and not found to differ significantly from that of the controls.

We wish once more to express our indebtedness to Professor Goodrich, F.R.S., in whose laboratory the work was done, and to the Empire Marketing Board and the Ministry of Agriculture and Fisheries for financial support.

Summary.

Winter Temperature Experiment.—The field mouse, when fed on summer food and allowed 15 hours of light each day, breeds less at low temperatures (about 5° C.) than at summer temperature. The fecundity of the males is not affected by the low temperature.

Winter Food Experiment.—The cutting down of fresh food to a minimum does not hinder reproduction.

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APPENDIX.

Table showing the conditions to which the winter temperature mice were exposed.

Month.	Mean temperature.	Mean relative humidity.
	° C.	
December, 1930	3.2	90.0
January, 1931	2.2	89.3
February, 1931	2.9	85.3
March, 1931	3.7	79.0
September, 1931	10.3	87.0
October, 1931	6.6	88.0
November, 1931	6.9	88.9
December, 1931	5.3	86.1

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The Inheritance by an Insect Vector of the Ability to Transmit a Plant Virus.

By H. H. STOREY, M.A., Ph.D. (Plant Pathologist, East African Agricultural Research Station, Amani).

(Communicated by F. T. Brooks, F.R.S.—Received July 27, 1932.)

The leafhopper, *Cicadulina mbila* Naude, is normally an efficient vector of the virus of streak disease of maize (Storey, 1925). I have, however, encountered exceptional individuals of this insect-species which were unable to transmit the virus (Storey, 1928). An attempt to discover the reason for their anomalous behaviour led me to undertake studies of the breeding of this species. The results so obtained, which show that the ability to transmit is an hereditary character, have been briefly reported (Storey, 1931), and I now present a full account of this work.

Since these studies introduce a new conception into pathology, I find it necessary to give my definition of certain current terms and to utilise two new ones. I employ *transmission* in the restricted sense of the act of transfer of a virus by a vector from a diseased to a healthy plant *in the natural process*

of that vector's feeding. I introduce the term *activity* to denote an insect's inherent* potentiality to transmit a virus; *inactivity* to denote the absence of that inherent potentiality. An insect is *viruliferous* or *non-viruliferous* according as it is, or is not, actually carrying a virus in such a way that it inoculates that virus into a plant on which it feeds, and in consequence causes the plant to develop the symptoms of the disease.

For clearness in the understanding of this account I may refer to the evidence which has been given elsewhere (Storey, 1928) that the leafhopper, *Cicadulina mbila*, is invariably non-viruliferous upon hatching from the egg; that thereafter the normal (*i.e.*, active) leafhopper of either sex and in any stage of its development may take up the virus by feeding on a diseased plant; and that the leafhopper, following its first feed upon a diseased plant, will normally become viruliferous after a latent period not exceeding 3 days at the temperatures at which my experiments have been performed, and will usually thereafter remain viruliferous without further feeding upon a diseased plant.

In the execution of this work Mr. R. F. W. Nichols has taken an important part, which I gratefully acknowledge. The early pure line breeding was carried out by him alone during my absence from Amani. In the later work he has undertaken the greater part of the manipulation of the experiments.

Materials.

In the first series of breeding experiments I used hoppers from a mixed culture of *Cicadulina mbila* which had been maintained for 2 years from parents originally collected near Tanga, Tanganyika Territory. From this mixed culture I isolated the pure lines designated *Act.* I and II and *Inact.* I and II. Later, further collections of hoppers were made in the same locality, and from these were bred the pure lines *Act.* IV and V and *Inact.* III and IV.

Preliminary evidence from the breeding of this species of leafhoppers indicated a falling-off of fertility in inbreeding. Consequently, in the course of the earlier breeding work now described, by crossing parallel lines whenever opportunity allowed I tried to avoid inbreeding. Later experience showed, however, that my fears of inbreeding were exaggerated; while crosses are probably more prolific than inbred lines, I have found it possible to rear lines successfully by inbreeding.

* The qualification "inherent" may appear tautological. A line of work now in hand indicates that the qualification is, on the contrary, essential.

Methods.

Two main considerations have determined the experimental procedure adopted in this work :

- (1) The test for the activity of a hopper rests upon whether it becomes viruliferous after feeding upon a diseased plant. A viruliferous leafhopper can be recognised as such only by studying the effect of its feeding upon a healthy plant.
- (2) The leafhopper lays its eggs in the tissues of a living leaf and these eggs will hatch only if the leaf remains alive through the period of their development.

The method used in the testing of families for activity was as follows. While still in an early nymphal instar, the hoppers were caged together upon a fully streaked leaf of a diseased maize plant. They fed upon the diseased leaf as long as was needed for them to reach a late nymphal instar, but never for less than 4 days. Thereafter each nymph was placed separately in a small single-leaf cage, of a design which I have described elsewhere (Storey, 1928), and allowed to feed upon the first leaf of a very young maize seedling until the disease symptoms appeared on that seedling—usually in 5 or 6 days—or, failing successful transmission, for a period of not less than 14 days.

A test carried out in this manner, as I have already shown (Storey, 1928), demonstrates with certainty whether a hopper of the species, *Vicatulinia mbila*, is viruliferous or non-viruliferous. I have also shown that it is usually safe to accept as inactive those hoppers which are non-viruliferous in such a test. Rarely, however, an active hopper, for some reason unknown, may fail to become viruliferous following the first period of feeding upon a diseased plant, and after a second feed on a diseased plant may become viruliferous. Consequently in many instances those hoppers which were non-viruliferous in the first test were returned to a diseased plant and then tested a second time. Usually I resorted to a second test only when the number of non-viruliferous hoppers was small in proportion to the number of viruliferous hoppers.

I have accepted the evidence of the second test as conclusive, since throughout this and earlier work I have encountered no instance where a hopper, later proved to be active, has failed to become viruliferous at least during the second feed on a diseased plant.

The test may fail if the hopper dies during the test. Generally a proportion of the hoppers in each family died. These deaths may be ascribed in part at least to injuries received in transfer to the cages, to drowning in water exuded into the cages from the hydathodes of the plant, and to difficulties encountered

in the final change of skin in the confined space of the leaf-cages. Many hoppers which died during the test nevertheless successfully inoculated the virus into the test plants. It would be unsafe to assume that hoppers which died during the test and failed to transmit were necessarily inactive. An uncertainty exists as to their status for the following reasons : (1) they may have been viruliferous, but may have died without feeding on the test plants, or (2) they may have been active but failed to become viruliferous after their first feed on a diseased plant, a situation which would have been revealed in a second test, had this been possible. To meet the former difficulty, in later work I adopted the procedure of discarding all individual tests in which the hoppers died during the first 2 days of the test, and excluding these tests from the totals. I assumed that no hopper would survive 2 days, under the conditions of my experiment, without feeding on the test plant. It is clearly impossible to confirm or disprove the possibility considered under the second of the preceding alternatives. The negative evidence which they provide must, however, at least be regarded as doubtful, and where it is in conflict with the mass of the evidence provided by the experiments in which it occurs, I have felt justified in not regarding it as significant.

The preceding paragraphs suggest that I have encountered considerable difficulties in the testing of families for activity. Actually, the exceptional behaviour of hoppers there considered has had a very small influence upon my results. The following figures are extracted from the records of the breeding of active races, which appear in Table I ; of 531 hoppers in families which I have regarded as active, 519 were viruliferous in the first test, 7 were non-viruliferous in the first test and viruliferous in the second test, while 5 died during the tests without causing infection of the test plants.

The tests were carried out in a gauze-protected greenhouse in which the maize plants were raised from seed. Two rows of 12 seeds each were sown in long narrow wooden boxes. One row of plants was usually retained as a control for the test plants. During the whole course of this work no control plant became diseased.

At the end of the test, the leafhoppers, which had been caged singly while still nymphs, had become adults and were ready for pairing. Each pair, selected as parents on the results of the tests, was confined in a glass tube, 20 cm. long by 25 mm. bore, closed at one end with muslin and at the other by a plug of cotton-wool. Through this plug was passed the tip of a leaf of a maize plant upon which the parent hoppers fed and laid eggs, and the newly hatched nymphs fed. The mean air temperature of the insectary

varied from 19° to 23° C. according to season, with absolute maxima and minima of 30° and 11° C. The breeding tubes were not exposed to direct sunlight except for a short period in the early morning. The hoppers bred at all seasons but more rapidly at the higher temperatures. At first difficulty was encountered through the high humidity and deposition of water within these tubes, which, apart from the direct ill-effects on the young nymphs, caused the premature death of the leaf-tip and consequently of many eggs laid in its tissues. The difficulty was overcome by a system of ventilation. An electric fan forced air into a main duct from which it was led into the glass breeding tubes by small rubber tubes passing through the wool plugs. A further development was the preliminary pairing of hoppers in small leaf cages, from which they were transferred to the breeding tubes only after egg-laying had begun. By these means the useful life of the leaf-tip in the breeding-tube was prolonged.

One further operation remains to be described. For the purpose of establishing bulked cultures of active races, I required to breed a non viruliferous progeny from parents, which, having demonstrated their ability to transmit in the tests, were necessarily viruliferous and would infect any plant to which they had access for the laying of their eggs. For this purpose the usual breeding procedure was followed, a leaf of an originally healthy plant being exposed within the breeding tube. The feeding of the parents produced no visible effect upon this leaf, although the plant as a whole became streak-diseased. I have shown that a hopper rarely picks up the virus from the green part of a diseased plant (Storey, 1928). Consequently by testing the young nymphs, which hatched in the tube, as early as possible upon maize seedlings, I was able to separate for bulking those generally a large proportion which had failed to become viruliferous.

The Breeding of Pure Lines.

Active Races.—The results of the first series of experiments in breeding from parents selected as active are presented in Table I. (This and subsequent Tables omit only those relevant pairings which produced no offspring. In certain instances both parents were selected from one family; but as far as possible the parents were selected from different lines, owing to my fear of a falling-off in fertility resulting from inbreeding. This fact makes the Table difficult to follow, but the manner of presentation allows the parentage of any family to be traced back through all its generations.

Of the nine F1 families raised, four showed all members active, though in three of these the numbers were small. Of the subsequent families raised

Table 1.—Breeding of Active Races.*

All parents selected as active.

Generation.	Family number.	Family number of parents.		Tests.	
		Female.	Male.	Number V.	Number N-V.
F1	A	From mixed culture		10	0
	B	"		22	2
	C	"		11	1
	E	"		55	0
	F	"		3	0
	G	"		21	1†
	H	"		25	8‡
	L	"		7	0
	N	"		19	6‡
F2	I	A	C	23	0
	II	A	C	10	0
	III	A	Mixed culture	17	0
	V	A	C	33	9‡
	IX	F	E	19	0
	XI	E	E	4	0
	XIII	E	E	5	2†
	XIV	E	E	8	0
	XVII	G	V (F2)	21	2
	XVIII	E	H	18	0
	XXI	L	20 (F3)	35	0
	XXII	L	20 (F3)	18	0
	XXIII	L	24 (F3)	10	2‡
	XXIV	N	N	11	6
	XXVII	N	N	31	0
	XXVIII	N	N	19	0
F3	1	II	I	24	0
	2	II	I	5	0
	6	I	II	7	1†
	7	I	I	19	0
	10	V	V	6	3‡
	14	V	H (F1)	20	3‡
	16	IX	2 (F3)	12	1†
	20	XVIII	XVIII	8	0
	24	XVIII	XVIII	14	0
	29	XXIII	XXIII	11	5
	31	XXIII	XXII	16	4
	32	XXIII	XXII	29	7
	33	XXIII	XXI	11	0
F4	a	1	2	7	0
	i	14	14	24	2‡
	q	7	7	9	0
	y	20	16	47	0
	b'	24	i (F4)	37	0
F5	BB	i	i	22	1
	DD	i	i	3	2
	EE	i	i	4	1†
	JJ	b'	b'	9	0
	MM	b'	b'	22	0

* In the Tables, viruliferous and non-viruliferous are designated by V and N-V.

† Hoppers died without second test.

‡ One or more of the non-viruliferous hoppers in each group received second test and proved

from parents selected from these F1 families, almost all came pure for activity. The exceptions are probably sufficiently explained by the death of hoppers during the tests, except in the instance of the F1 family No. I, the subsequent history of which in the F2 and F3 generations (see XXIII and 29, 31, 32) shows that it was not pure for activity, although the seven hoppers of which it consisted were all active.

Five of the F1 families contained both active and inactive hoppers. Parents selected as active from these mixed families gave in some instances pure progenies and in some instances mixed. Thus in the F2 generation, crossings of parents from C (mixed) and A (pure) gave two pure progenies and one mixed progeny. Inbreeding within the family N (mixed) gave two pure progenies and one mixed.

The breeding was carried on to the fifth generation, although it is clear that for the mere purpose of obtaining pure lines the work might have been stopped earlier. In starting bulked cultures of pure lines I assumed that I might accept a line as pure if parents, taken from apparently pure families, produced a progeny similarly pure. The following bulked cultures were thus established :—

Act. I.—Parents from IX, XI, (F2) and I (F3).

Act. II.—Parents from γ (F4).

Further bulked cultures, Nos. *Act. IV* and *V*, were established by breeding from female hoppers, caught as adults in the field. A small proportion of these females gave progenies which proved to be apparently pure for activity. The F2 inbred generation from these was tested and bulked. The details of the families appear in Table II.

The bulked cultures have been maintained for a considerable period. *Act. I* and *II* since October 23, 1930 and March 2, 1931, respectively. Table III shows the results of periodical sampling tests of certain cultures. These tests indicated a satisfactory maintenance of purity, with the exception of the one test of culture *Act. I* on April 11, 1931. The 10 non-viruliferous hoppers in this test all died before a second test could be made. It will be noticed that in a test of the same culture at a later date all hoppers were active.

In the breeding-up of large numbers of active hoppers, I have found it advantageous to make uncontrolled crossings between the several bulked pure lines. These crossings have resulted in cultures pure for activity, as the sample tests in Table III demonstrate.

Table II.—Breeding of Active Races.

Races inbred from wild active parents.

Race number.	Generation.	Family number.	Tests.	
			Number V.	Number N-V.
<i>Act. IV</i>	F1 F2	IV	47	0
		IV.1	21	0
		IV.2	5	0
		IV.4	12	0
		IV.6	14	0
		IV.7	5	0
		IV.8	10	0
		IV.9	9	1*
		IV.10	19	0
<i>Act. V</i>	F1 F2	V	26	1*
		V.1	16	1*
		V.3	6	0
		V.6	13	0

* Hoppers died without second test.

Table III.—Sample Tests of Bulkcd Active Cultures.

Sample of hoppers taken from culture, fed for few days on streak-diseased maize and tested singly.

Culture number.	Date of test.	Tests.	
		Number V.	Number N-V.
<i>Single Lines -</i>			
<i>Act. I</i>	December 31, 1930	29	1*
	April 11, 1931	66	10*
	October 23, 1931	18	1*
	April 30, 1932	35	0
	May 15, 1931	15	0
<i>Act. II</i>			
<i>Crossed Lines -</i>			
<i>Act. I</i> × <i>Act. IV</i>	March 26, 1932	25	0
<i>Act. II</i> × <i>Act. IV</i>	March 26, 1932	20	0

* Hoppers died without second test.

Inactive Races.—The results of breeding for inactivity are summarised below :—

Parents—Selected as inactive, from original mixed culture.

F1.—Six families raised, averaging 36 hoppers per family—All inactive.

F2.—Ten families raised, averaging 9 hoppers per family—All inactive,

F3. --Eleven families raised, averaging 13 hoppers per family. All inactive, except 2 hoppers, one in each of two families.

F4. -- Six families raised, averaging 11 hoppers per family. All inactive, except 1 hopper.

F5. --Ten families raised, averaging 11 hoppers per family. All inactive.

F6. --Five families raised, averaging 19 hoppers per family. All inactive.

The breeding from inactive parents thus resulted in progenies, which were entirely inactive, except that two families in the F3 generation and one in the F4 generation each contained one active hopper. In the two former instances the activity of the hoppers was confirmed in a second test. It seems impossible at present to explain these anomalous individuals except on the grounds of experimental error, for which in the necessarily complicated technique there is some opportunity. Whatever be the true explanation, however, these few exceptions -- 3 in 700 -- do not invalidate the general conclusion that lines may be bred true for the factor of inactivity.

In the course of the preceding pure line breeding, I established two bulked inactive cultures, designated *Inact.* I and II. Two more bulked cultures, *Inact.* III and IV, were started by inbreeding from wild females collected in the field, as shown in Table IV. It will be seen from this Table that the original female parents were active, but gave mixed F1 progenies. In the instance of line III, I was able to select from the F1 family inactive males and females, which when paired gave progenies pure for inactivity. The line IV gave an F1 family in which all the surviving inactive members were males, so that it

Table IV. Breeding of Inactive Races.

Races bred from wild parents.

Race number.	Generation.	Family number.	Parents.		Tests.	
			Female.	Male.	Number V.	Number N-V.
<i>Inact.</i> III	F1	III	Active	?	7	13
	F2	III.1	Inactive	Inactive	0	6
		III.3	Inactive	Inactive	0	12
	F3	III.1.1	Inactive	Inactive	0	23
		III.3.1	Inactive	Inactive	0	37
<i>Inact.</i> IV	F1	IV	Active	?	26	43*
	F2	IV.4	Active	Inactive	11	10
	F3	IV.4.1	Inactive	Inactive	0	22
	F4	IV.4.1.1	Inactive	Inactive	0	24
		IV.4.1.2	Inactive	Inactive	0	24

* Surviving non-viruliferous hoppers, all males.

was necessary to pair these with active females of the same family. The resulting F₂ family was consequently mixed, but parents selected as inactive from this family gave pure inactive progenies in the F₃ and F₄ generations.

Sample tests of these bulked cultures and of certain crossed bulked cultures invariably revealed a maintenance of purity for inactivity, as shown in Table V.

Table V.—Sample Tests of Bulked Inactive Cultures.

Sample of hoppers taken from culture, fed for a few days on streak-diseased maize and tested singly.

Culture number.	Date of test.	Tests.	
		Number V.	Number N-V.
<i>Inact.</i> I	October 22, 1931 . . .	0	27
	November 19, 1931 . .	0	27
	April 23, 1932	0	33
	May 15, 1932	0	33
	March 18, 1931	0	65
<i>Inact.</i> II	April 11, 1932	0	13
<i>Inact.</i> I × <i>Inact.</i> III	April 11, 1932	0	20

The Crossing of Pure Lines.

The results of the two reciprocal crosses between the pure active and inactive races are shown in Tables VI and VII. It will be seen that the cross

Table VI.—Crosses—Active Female × Inactive Male.

Generation.	Family number.	Parents.		Females.		Males.	
		Female.	Male.	Active.	Inactive.	Active.	Inactive.
F ₁	A	Active	Inactive	15	0	14	0
	B	Active	Inactive	13	0	5	0
	C	Active	Inactive	18	0	21	0
			Totals.....	46	0	40	0
			Expectation	43	0	43	0
F ₂	A.1	Active	Active	5	0	1	4
	A.2	Active	Active	8	0	4	2
	B.1	Active	Active	5	0	4	1
	B.2	Active	Active	6	0	3	3
			Totals	24	0	12	10
			Expectation	23	0	11.5	11.5

Table VII. -- Crosses -- Inactive Female \times Active Male.

Generation.	Family number.	Parents.		Tests.			
		Female.	Male.	Females.		Males.	
				Active.	Inactive.	Active.	Inactive.
F1	A	Inactive	Active	27	0	0	14
	B	Inactive	Active	3	0	0	7
	C	Inactive	Active	8	0	0	14
	D	Inactive	Active	17	0	0	23
	E	Inactive	Active	27	0	0	19
		Totals		82	0	0	77
F2		Expectation		79.5	0	0	79.5
	A.1	Active	Inactive	8	11	8	7
	A.2	Active	Inactive	1	1	1	1
	A.3	Active	Inactive	0	1	1	0
	A.4	Active	Inactive	0	0	3	3
	A.5	Active	Inactive	7	9	12	1
	A.6	Active	Inactive	2	4	3	0
	A.7	Active	Inactive	4	2	1	0
	A.8	Active	Inactive	1	5	0	1
	A.9	Active	Inactive	0	3	4	4
	B.1	Active	Inactive	4	5	2	3
	B.2	Active	Inactive	2	2	3	3
		Totals		29	43	38	23
		Expectation		33.25	33.25	33.25	33.25
Omitting family A.5		Totals		22	34	26	22
		Expectation		26	26	26	26

active female \times inactive male gave an entirely active progeny in the F1 generation. The F2 generation of this cross consisted of active females and both active and inactive males. The reciprocal cross, inactive female \times active male, resulted in the F1 generation in only active females and inactive males. Both active and inactive males and females appeared in the F2 generation.

I offer the hypothesis that in the species *Cicadulina mobile* the male is heterozygous for sex, that the factor for activity is dominant to that for inactivity and that this factor is linked with sex (in the manner exemplified in the well-known instance of *Drosophila*); that is (employing the conventional notation), that the inactive male has the constitution (aX) (Y), the active male (AX) (Y), the inactive female (aX) (aX), and the active female either (AX) (AX) or (AX) (aX). This hypothesis requires in the several generations of crosses the ratios shown opposite "Expectation" in Tables VI and VII. An examination

of the figures in these tables shows a close agreement of the observed totals with the expectation. In particular, it will be noted that the hypothesis requires that in the F1 and F2 generations of Table VI and the F1 of Table VII certain categories shall not appear in the families. In every instance this requirement has been realised. Thus, for example, the hypothesis requires that the F1 generation in Table VII shall contain no inactive females or active males; these categories were absent from the five independent families reared.

The closeness of the agreement of the observed totals with the expectation is obvious by simple inspection in all instances except in the F2 generation of the cross inactive female \times active male (Table VII). The χ^2 test applied to the totals of this generation gives a value of $P = 0.065$, which falls just above the value ($P = 0.05$) generally accepted as implying a significant fit to the expected ratios. If the individual families be examined, all show a significant fit to the expected ratios (family A8 barely so), except family A5, which shows a wide deviation from the expectation. I am unable to explain this anomalous result. If it may be regarded as due to experimental error, the totals of the remaining 10 families show a reasonably close fit to the expected ratios ($P = 0.3$ approximately).

Comparison of Active and Inactive Races.

There can be little doubt that both active and inactive races judged by the usual criteria of the systematist fall into the one species *Cicadulina mbila*. I have failed to find any point of difference in the external morphology of members of the two races. Mr. T. W. Kirkpatrick, Entomologist of the East African Agricultural Research Station, has confirmed my conclusion after an independent examination. In particular we find no difference in the male genitalia of the races, which agree with the figures of China (1928).

Crosses between the two races, as has already been noted, are fertile.

Both races feed freely on a maize leaf and I have observed no difference in their length of life. Sections of maize leaves, upon which the two kinds of hoppers have fed, show microscopically no difference in the character of the feeding channel. The feeding stylets of the two races are not detectably different in average length. Nevertheless, although the active race of hoppers usually became viruliferous after a short period of feeding upon a diseased maize leaf under my experimental conditions, the inactive race, as the evidence already given shows, invariably failed to become viruliferous. The same is true at temperatures outside the range encountered in that work. In an experiment, groups of about 20 inactive hoppers were each fed on diseased

maize for 2 days at temperatures of 10° C., about 24° C. (room temperature), 30° C. and 40° C. Only five hoppers survived the highest temperature. No hopper in any series became viruliferous. Similar failures were encountered in every instance where the period of feeding on diseased maize was reduced to 1 hour and extended to several days.

Discussion.

The possibility of a differing ability to transmit a virus in different races of one vector-species has probably been considered by many workers who have studied insect-transmission. K. M. Smith (1931, *a*), for example, has recently drawn attention to this possibility. Hoggan (1929-1931) in her work upon the vector-species, *Myzus persicae* Sulz., took special precautions to avoid errors through the use of a single strain of the aphids. I believe, however, that the work now described provides for the first time a definite demonstration that strains of unequal ability to transmit a virus may exist within a single species.

This phenomenon is possibly of a more general occurrence than has yet been shown. It is true that Kunkel (1926) decided that, in the species *Cicadulina sexnotata* Fall., "most, if not all, individuals are capable of taking up the aster yellows virus." This conclusion, however, is based upon the study of only 30 hoppers, whose origin, although not clearly stated by Kunkel, may have been an inbred line. On the other hand, unexplained failures are a common feature of the majority of published descriptions of experiments in the insect transmission of plant viruses. The factors concerned in these failures are doubtless highly complex; of these the physiological condition and genetical constitution of the experimental plants are not the least important. Of the factors which concern the vector comparatively little is known, although definite advances have been made in the demonstration of a latent period of the virus in the insect (Carsner and Stahl, 1924; Kunkel, 1926; Linford, 1932; Severin, 1921; Smith, 1931; Storey, 1928), of the necessity for certain vectors to take up the virus in a particular stage of their development (Linford, 1932; Samuel and Bald 1931), and of the loss of the ability to inoculate the virus in the ageing of certain vectors (Kunkel, 1926; Storey, 1928). A further insight into the part played by the vector is now afforded by the proof that hereditary factors may determine the ability of an insect to transmit a virus.

The conclusion that I have bred races of *Cicadulina mbila* which, in the natural process of feeding, are unable to transmit the virus of streak disease, can hardly be doubted. Under exactly parallel conditions the active races have transmitted, while the inactive races have failed. Under abnormal

conditions as to length of the period of feeding on a diseased plant and the temperature at which this feeding takes place, the inactive races have failed equally. It is true that my method of testing might lead to erroneous conclusions, if the difference between the active and inactive races lay in a prolonged latent period of the virus in the latter. I consider, however, that my evidence excludes this possibility. In many instances the period of observation of the test plants has extended over considerably more than the minimum of 14 days. Where a second test has been carried out upon a supposed inactive hopper, the period of observation has extended over more than 5 weeks from the first exposure of the hopper to the virus. Conclusive evidence, however, is provided by the bulked cultures, in which supposed inactive hoppers, after a period of feeding on a diseased plant and a second period of testing, were caged for the rest of their lives on maize plants. In no instance did the plants develop the disease. It is improbable that the difference between the active and inactive races lies in a rapid loss of the ability to inoculate the virus, after the manner of that reported by Kunkel (1926) for *Cicadula sexnotata*, since in certain instances the period of feeding on a diseased plant, preliminary to the test, has been reduced to as short a period as 1 hour, without causing any inactive hoppers to give evidence of being viruliferous.

Studies are now in progress in an attempt to discover the basis of the difference in behaviour of the two races. At this stage I may say that by certain treatments I have been able to obtain viruliferous hoppers from an inactive race. I have, however, no evidence to cause me to doubt the truth of the statement that, *in the natural process of feeding*, the inactive race hoppers will invariably fail to transfer the virus of streak disease.

Summary.

(1) Races have been bred of the species, *Cicadulina mbila* Naude, which are on the one hand able, and on the other unable to transmit the virus of streak disease in the natural process of feeding on maize plants.

(2) The crossing of the pure races has demonstrated that the ability to transmit is inherited as a simple dominant Mendelian factor, linked with sex.

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Narcosis and Oxidations of the Brain.

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A form of therapy frequently used in the treatment of mental disorder which, in spite of its dangers, merits further study and investigation, is that of prolonged narcosis. A period of 12 to 14 days is not uncommonly used and there is no question but that, in many cases, psychotic symptoms are alleviated or their development checked. An adequate explanation of this phenomenon is still wanting, and it is evident that a knowledge of the mechanism of narcosis itself is necessary for a full comprehension of the logical basis of narcotic treatment in mental disease.

In spite of the immense amount of research which has been carried out on narcotics and anaesthetics there is no clear evidence yet as to what physiological or metabolic disturbances occur which result in narcosis. No attempt will be made here to deal with the extensive literature which has accumulated and which has been admirably summarised by Winterstein (1926) and Henderson (1930). There must still be agreement with Cushny (1924) who says "after the narcotics have penetrated into the brain cell, the effects depend on some further quality which is still unknown."

Little doubt exists that metabolic changes in the cell are influenced by adsorption of narcotics either at the cell surface or at intracellular structures,

the nature of the lipoids present influencing to a considerable extent the quality, the amount and the site of adsorption. Experiment, however, has yet to show what specific metabolic activities are affected and what connection may lie between such disturbances in the metabolism of the cell and its changes in functional activity.

Verworn's asphyxial theory of narcosis was inadequate to explain many phenomena connected with narcosis and was certainly not in harmony with facts which showed that narcosis could be induced in cells leading an anaerobic existence. Henderson (1930) goes so far as to say that "no theory of anæsthesia will prove acceptable which is based on a proof of a depression of the resting oxidation of the cell . . ." "The facts distinctly show that oxidative processes and narcosis are separate phenomena." These statements, in view of the insufficient evidence, are too sweeping. The oxidations of a cell are manifold and its resting oxidation is composed of numerous processes. One process may be hindered by narcotics and others may be unaffected. Clearly a change in resting oxidation due to a narcotic can give little conception of the magnitude of the effect of the drug on any specific metabolic change and in this sense it is true to say that a theory of the mechanism of narcosis cannot be based on studies of resting oxidation alone. It cannot be denied, however, that certain metabolic processes, vital to the specific functional activities of the cell, may be influenced profoundly by the presence of narcotics, whilst the changes of the cell taken as a whole may not be appreciably affected.

It is hoped to demonstrate in this communication that narcotics do not influence the activation of oxygen by the brain cell, nor do they affect the oxidation by the cell of certain substances which are freely oxidised by many cells which live aerobically. It will be shown, however, that narcotics exert a profound inhibitory action on the oxidation by the brain of substances important in carbohydrate metabolism, viz., glucose, lactic acid and pyruvic acid—this action being accomplished at concentrations of narcotics which are of the same order as those which give rise to deep narcosis in animals. This behaviour is not due to a disturbance of the cell whereby its mechanisms for accomplishing oxidations are affected—but to an effect, possibly at cell interfaces, whereby the mechanism, which results in an activation of the molecules of lactic and pyruvic acids, is greatly disturbed. Such an interpretation of the behaviour of a narcotic serves not only to explain phenomena observed in the study of narcosis in animal life but to explain the fact that narcosis may be induced in anaerobic cells.

Experimental Technique.

Attempts have been made by the writers (1932) to acquire data concerning brain oxidations whereby it would be possible to judge the normality or the abnormality of a particular brain tissue. Such information would be very valuable, especially in circumstances where histological evidence failed to show any appreciable differences between normal and abnormal tissue. The work was initially attended with some difficulty owing to the variability in the rates of oxygen uptake in the freshly excised tissues, owing, in all probability, to variations in the initial quantities of lactic acid in the brain. On discovering, however, that the presence of lactic acid had considerable influence a "sparing" action—on the oxidation of a metabolite such as succinic acid the technique was so modified, that the amount of lactic acid in the brain was greatly diminished before observations of the O_2 uptake in the presence of certain substrates were determined. Consistent and reliable results were then obtained. The modification of the original method simply consisted in allowing the fresh brain tissue to take up oxygen, whilst being shaken in a vessel of a Barcroft respirometer, until the velocity of uptake had fallen to between half and two-thirds of its initial value. This took usually about 3 hours, after which the substrates under investigation were added and the subsequent rates of O_2 uptake were measured. Descriptive results are shown in fig. 1 where the tissue consisted of 0.5 gm. of whole guinea-pig brain. Curve A indicates the effect of adding glucose (0.025 per cent. in saline) to

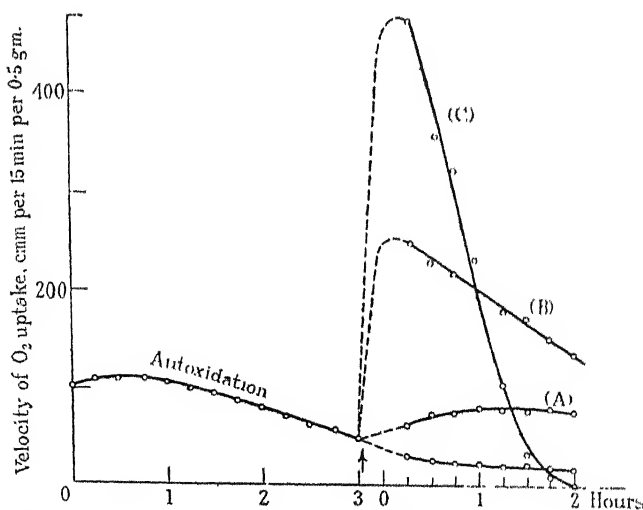


FIG. 1.—Curve A, glucose; B, succinate (0.05M); C, *p*-phenylenediamine (15 mg)
D, autoxidation,

the brain tissue after 3 hours prior oxidation and it will be noted that there is a rapid rise in O_2 uptake almost to the initial level and this level is kept constant for a considerable period. Clearly little injury has been done to the brain cells during the period of 3 hours in which they were shaken in the Barcroft vessel prior to addition of the sugar. The constant level of the rate of O_2 uptake due to glucose is of great interest when compared with the effects of adding sodium succinate (curve B) and *p*-phenylenediamine (curve C). In these cases there is a most rapid increase in the rate of O_2 uptake followed by a rapid fall, showing an intensity of oxidation far greater than that of glucose, whose breakdown is accomplished in a more leisurely but nevertheless much more steady and constant manner. Sodium lactate and sodium pyruvate are oxidised by the brain in a manner almost identical with that of glucose and for equivalent concentrations the curves of the rates of O_2 uptake due to the three substances cannot be distinguished from each other. Such a fact supports the conclusion (for which Holmes (1925) has given evidence) that in the brain, glucose oxidation takes place via the intermediate formation of lactic acid, and this is also supported by the observation of the writers (1932) that the addition of glucose to lactic acid in the brain does not bring about an increased rate of oxygen uptake. There are, however, certain difficulties still in the way of complete acceptance of this view; these are referred to in detail in a previous paper (Quastel and Wheatley, 1932) and will not be discussed here. Full details of the experimental technique employed in the experiments about to be described are given in that paper. It will suffice here to say that 0.5 gm. mixed (whole) brain tissue was used in the case of all small animals, grey matter of cortex only being used with large animals. When estimating the effects of narcotics, the drug under investigation was dissolved in saline solution and added to the brain tissue at the commencement of the experiment, phosphate solution (M/15 at pH 7.4) being used as a buffering medium. Control experiments were always carried out. Measurements of oxygen uptake were made at 15-minute intervals, the temperature of the bath in which the Barcroft vessels containing the tissue were shaken being 37°. Except where otherwise stated air was used in all vessels.

Action of Somnifaine on the Oxygen Uptake of Brain Tissue.

Somnifaine, a drug composed of the diethylamine salts of veronal and allylisopropylbarbituric acid (10% of each in glycerol-water-alcohol solution), has been used somewhat extensively in the treatment of certain types of mental disorder. Its hypnotic powers are much greater

than those of veronal and it was considered a suitable substance for an investigation into the effects of narcotics on brain tissue *in vitro*. A solution of it in saline was neutralised, and added to 0.5 gm. brain tissue contained in a Barcroft manometer vessel, to reach a final concentration of 0.4 per cent. A powerful inhibitory action was exercised by the drug on the autoxidation of fresh brain tissue at this concentration. The initial relatively high level of oxygen uptake was reduced, in certain cases, by over 90 per cent. Typical results are shown in Table I.

Table I.—Action of Somnifaine (0.4 per cent.) on O_2 uptake of Brain (0.5 gm.) in 90 Minutes.

Animal	Normal.	In presence of somnifaine.
	c.mm.	c.mm.
Guinea-pig	585	55
Cat (grey)	422	40
Rabbit	431	70
Mouse	480	140
Calf (grey)	591	145
Sheep (grey)	331	120
Man (grey)	376	56

In addition to the brain tissues of animals quoted in Table I, those of pigeon, tortoise, frog and rat were also found to be greatly affected by the presence of somnifaine. The inhibitory action of the drug is also very marked at low concentrations, *e.g.*, with guinea-pig brain, a 0.1 per cent. solution of somnifaine brought about a 40 per cent. inhibition of oxygen uptake. This concentration of somnifaine is about that required to induce deep narcosis in the guinea-pig.

Control experiments showed that diethylamine had but little inhibitory action on the oxidation of brain and it could be easily shown that allylisopropylbarbituric acid exercised a far greater effect than diethylbarbituric acid (veronal).

Brain and Yeast.

The next step was to determine whether the inhibitive action of the dialkylbarbituric acid derivatives took place with any actively respiring tissue. Investigations with yeast showed that its oxygen uptake was in no way inhibited by concentrations of allylisopropylbarbituric acid and of ethylurethane which were effective with brain (Table II).

Table II.—Action of Narcotics (0·3 per cent.) on O₂ uptake (in 2 hours) of Yeast and Brain.

Narcotic.	Yeast (0·05 gm.).	Brain (0·5 gm.).
	c.mm.	c.mm.
Allylisopropylbarbituric acid	290	492
Ethylurethane	251	670
No narcotic	252	840

There is little question of the inhibitive effects of the narcotics on yeast respiration at very high concentrations but at those concentrations which are of the order used in inducing narcosis in animals and which affect the respiration of brain, the oxidation of yeast is either slightly or not at all disturbed. Further investigations have shown that brain tissue shows greater susceptibility (so far as its O₂ uptake is concerned) to narcotics than muscle, kidney or liver tissue. Results with liver tissue, however, were very variable—in contrast to those with brain—the effects being dependent on the state of nutrition of the animal. The investigation of the effects of narcotics on liver oxidations will form the subject of another communication.

The Action of Dialkylbarbituric Acid Derivatives on Oxidations of the Brain.

The question arose as to whether narcotics of the dialkylbarbituric acid series inhibited in a general manner all oxidations of the brain or whether they affected the oxidations in any specific manner.

To settle this question it was necessary to adopt the modified technique briefly described above. Brain tissue, in the presence of narcotics, was allowed to take up oxygen for 3 hours until the rate of uptake had fallen to between half and two-thirds of its initial value and metabolites were then added and the subsequent rates of O₂ uptake were measured. Control experiments (in the absence of narcotics) were carried out.

Typical curves illustrating the action of allylisopropylbarbituric acid (A.I.B.) on the autoxidation of guinea-pig brain, and of the effect of the addition of glucose (0·025 per cent. in saline) to the narcotised brain, are shown in fig. 2. An examination of the curves shows the following points of interest:—

1. A rise in the rate of oxygen uptake by normal brain and brain treated with A.I.B. to a maximum within 1 hour followed by a linear decrease in the rate of oxygen uptake for 3 hours when the curve flattens and the rate begins to fall off very slowly.

2. A lowered rate of oxygen uptake due to the action of A.I.B. The curves of oxygen uptake of the normal and treated brains cross, however, so that after 4 hours the rate of oxygen uptake with the narcotised brain is actually greater than that of the normal.

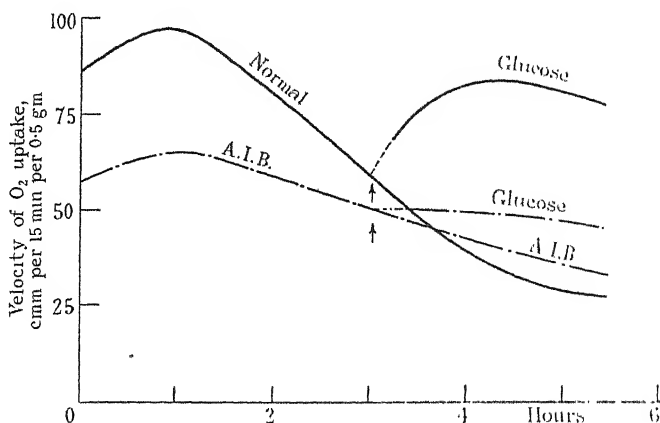


FIG. 2.

3. A considerable rise in oxygen uptake due to addition of glucose to the normal brain after 3 hours' autoxidation, and a relatively small rise due to addition of glucose to the narcotised brain.

The action with sodium lactate is in every way similar to that with glucose, there being no doubt but that there is a very marked lessened ability of the narcotised brain to oxidise glucose and lactate.

The crossing of the two curves is, probably, due to the initial content of lactic acid being utilised far more quickly in the case of normal brain than in that of the narcotised brain, so that the rate of fall of oxygen uptake is greater in the former case than in the latter.

The behaviour of the normal and treated brains is much the same towards serum as it is towards glucose or lactate, fig. 3; this, probably, is due to the presence in the serum of both glucose and lactate.

Quite opposed to this behaviour, however, is the effect of narcotised brain on sodium succinate and *p*-phenylenediamine. Here an entire lack of inhibitive action is evinced. This will be discussed later.

Relation between the Chemical Constitution of Dialkylbarbituric Acid Derivatives, their Inhibitive Action on Brain Oxidations and their Hypnotic Activities.

A series of soluble dialkylbarbituric acid derivatives, whose constitutions differed only in the nature of the alkyl radicles attached to the malonyl residue,

was investigated. It was found to be a matter of considerable importance when attaching significance to any relationship between constitution and inhibitory action, to use substances whose properties were such that they could be fairly compared when used under identical physical conditions, *e.g.*, that they were completely soluble at the p_H (7.4) of experiment and at the concentration (0.12 per cent.) found to be suitable for investigation. Through the kindness and courtesy of Dr. M. Guggenheim (of the firm of Hoffmann-La Roche) who has placed a number of dialkylbarbituric acid derivatives at our disposal, it has been possible to investigate a number of derivatives whose physical characteristics are such that it is possible to make a comparison

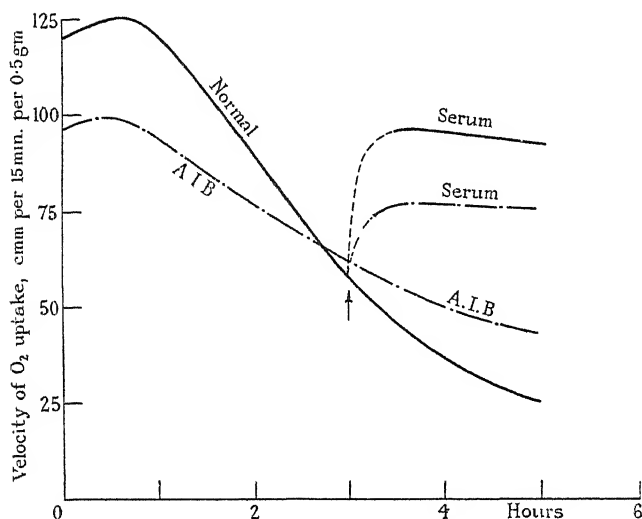


FIG. 3.

between their constitutions and their effects upon the brain. Many of the derivatives, however, could not be used with profit owing to their relative insolubilities under the conditions of these experiments.

The effects of eight dialkylbarbituric acid derivatives on the autoxidation of guinea-pig brain and on the oxidation of glucose by brain, after this had been partially depleted of oxidisable material by previous oxidation for 3 hours, are noted in Table III. The derivatives were dissolved, and made neutral, in saline solution and added to the brain tissue at the commencement of experiment to give a final concentration of 0.12 per cent. The percentage inhibitions of autoxidation and of glucose oxidation effected by the substances are shown in this table. The relative hypnotic activities of the derivatives are also noted. These are expressed by the symbols : 0 which represents lack

of hypnotic action, + which represents a weak activity, and +-+ which represents a relatively high hypnotic activity.

It seems scarcely feasible to make more quantitative representations of the hypnotic powers of the derivatives than those given. This is owing to the fact that hypnotic activity varies quantitatively from animal to animal and according to the method of administration. Moreover, it is difficult to express hypnotic activities in such a way that they can be compared quantitatively with the percentage inhibitions of oxidations by brain effected by a particular concentration of narcotic. Accordingly the representations given above have been adopted and these, shown in Table III, are a fair estimate of the relative hypnotic powers of the derivatives in question.

Table III.

Derivative (0.12 per cent.).	Percentage inhibition of autoxidation.	Percentage inhibition of O ₂ uptake in presence of glucose.	Hypnotic action.
Isopropylbarbituric acid	10(0)	6	0
$ \begin{array}{c} \text{CH}_3 \quad \text{CO-NH} \\ \diagdown \quad \diagup \\ \text{C} \\ \diagup \quad \diagdown \\ \text{CH}_3 \quad \text{CO-NH} \quad \text{CO} \end{array} $			
Isopropylbarbituryl urethane	9	4	0
$ \begin{array}{c} \text{CH}_3 \quad \text{H} \\ \diagdown \quad \diagup \\ \text{C} \\ \diagup \quad \diagdown \\ \text{CH}_3 \quad \text{C} \quad \text{CO-NH} \quad \text{CO} \\ \diagup \quad \diagdown \quad \diagup \quad \diagdown \\ \text{C}_2\text{H}_5\text{O-CO-NH} \quad \text{CO-NH} \end{array} $			
Isopropylbrompropenylbarbituric acid (noctal)	40	50	+-+
$ \begin{array}{c} \text{CH}_3 \quad \text{H} \\ \diagdown \quad \diagup \\ \text{C} \\ \diagup \quad \diagdown \\ \text{CH}_3 \quad \text{C} \quad \text{CO-NH} \quad \text{CO} \\ \diagup \quad \diagdown \quad \diagup \quad \diagdown \\ \text{CH}_2=\text{CBr} \cdot \text{CH}_2 \quad \text{CO-NH} \end{array} $			
Isopropylbrompropylbarbituric acid	9	0	Very weak
$ \begin{array}{c} \text{CH}_3 \quad \text{H} \\ \diagdown \quad \diagup \\ \text{C} \\ \diagup \quad \diagdown \\ \text{CH}_3 \quad \text{C} \quad \text{CO-NH} \quad \text{CO} \\ \diagup \quad \diagdown \quad \diagup \quad \diagdown \\ \text{CH}_3\text{CH Br} \cdot \text{CH}_2 \quad \text{CO-NH} \end{array} $			

Table III—(continued).

Derivative (0.12 per cent.).	Percentage inhibition of autoxidation.	Percentage inhibition of O ₂ uptake in presence of glucose.	Hypnotic action.
Isopropylallylbarbituric acid (numal)	34	40	++
$ \begin{array}{c} \text{CH}_3 \quad \text{H} \\ \diagdown \quad \diagup \\ \text{C} \\ \diagup \quad \diagdown \\ \text{CH}_3 \quad \text{C} \quad \text{CO-NH} \\ \diagdown \quad \diagup \quad \diagdown \quad \diagup \\ \text{CH}_2=\text{CH} \cdot \text{CH}_2 \quad \text{CO-NH} \quad \text{CO} \end{array} $			
Phenylallylbarbituric acid ..	50	57	++
$ \begin{array}{c} \text{C}_6\text{H}_5 \quad \text{CO-NH} \\ \diagdown \quad \diagup \\ \text{C} \\ \diagup \quad \diagdown \\ \text{CH}_2=\text{CH} \cdot \text{CH}_2 \quad \text{CO-NH} \quad \text{CO} \end{array} $			
Phenylethylbarbituric acid (luminal)	33(72)	40	++
$ \begin{array}{c} \text{C}_6\text{H}_5 \quad \text{CO-NH} \\ \diagdown \quad \diagup \\ \text{C} \\ \diagup \quad \diagdown \\ \text{C}_2\text{H}_5 \quad \text{CO-NH} \quad \text{CO} \end{array} $			
Diethylbarbituric acid (veronal)	15(50)	10	+
$ \begin{array}{c} \text{C}_2\text{H}_5 \quad \text{CO-NH} \\ \diagdown \quad \diagup \\ \text{C} \\ \diagup \quad \diagdown \\ \text{C}_2\text{H}_5 \quad \text{CO-NH} \quad \text{CO} \end{array} $			

() — Human brain (grey matter).

The following points of interest may be noted :—

1. Whereas isopropylbarbituric acid is almost inert both as a hypnotic and as an inhibitor of oxidations, the isopropylallyl derivative is intensely active.
2. The combination of isopropylbarbituric acid with urethane may have been expected to produce a compound of considerable hypnotic activity. The reverse is the case, the urethane-isopropyl derivative of barbituric acid being inert so far as hypnosis is concerned and practically so, so far as its effects upon brain oxidation are concerned.
3. The introduction of a bromine atom into the allyl radicle of allyl-isopropylbarbituric acid neither reduces hypnotic activity nor the inhibitive

effect upon oxidations. The reduction, however, of the unsaturated linkage of the brom derivative to isopropyl-brompropyl barbituric acid greatly reduces the hypnotic activity and, at the same time, its power to inhibit oxidations of the brain.

There must be many factors which determine whether or not a certain derivative has hypnotic activity, *e.g.*, speed of fixation by the tissues, speed of elimination or breakdown in the body, specificity of distribution in the tissues, and these must be greatly dependent upon purely physico-chemical characteristics. It is difficult to find any exact general relationship between physico-chemical properties, on the one hand, and the chemical structure of the various dialkylbarbituric acids, on the other hand, and it is therefore still more difficult to find a relationship between chemical structure and pharmacological activity which is greatly dependent on physico-chemical properties.

It may be stated, however, from the experiments quoted above, that there is a marked parallelism between the hypnotic activity of the drug and its ability to inhibit the oxidations of brain, when using dialkylbarbituric acid derivatives whose physical properties are such that they can be compared quantitatively under the conditions of these experiments. There is no *linear* proportionality and this could not be expected considering the many factors influencing the distribution of the drug in the animal organism, which do not obtain under our experimental conditions. Yet the parallelism which is found can scarcely be fortuitous and would lead to the conclusion that there must be some relationship or mutual dependence between the power of the drug to inhibit some particular oxidation by the brain and its hypnotic activity.

Using human grey matter, a much greater sensitivity towards the dialkylbarbituric acid derivatives is evinced (some figures for human material are quoted in brackets in Table III), but owing to the difficulty of obtaining fresh human material, it has been impossible, so far, to acquire much reliable information.

It is to be anticipated that, if there is a dependence between hypnotic activity of a drug and its effect on oxidations of the brain, this phenomenon will be shown not only by narcotics of the barbituric acid series but by narcotics of quite different chemical constitution. This is so: hypnotics such as hyoscine, chloral hydrate, chloretone, are found to possess powerful inhibiting effects upon oxidations of the brain.

Let us now enquire further into the specificity of attack by narcotics on brain oxidations.

Specificity of Attack by Narcotics on Brain Oxidation.

Using the methods already described it has been found that the narcotics inhibit the oxidations by brain of glucose, sodium lactate and sodium pyruvate. They have no inhibitory action on the oxidation of sodium succinate or *p*-phenylenediamine at the concentrations used. Table IV shows the percentage inhibitions of extra oxygen uptakes due to these substances effected by a series of narcotics. Figures expressing actual amounts of oxygen consumed by these substances under normal conditions are given in a previous paper (Quastel and Wheatley, 1932). The inhibitive effects of the narcotics are not materially affected by the presence of serum.

Table IV.—Percentage Inhibition by Narcotics of extra Oxygen Uptakes due to various Substrates.

Narcotic (0·12 per cent.)	Glucose. (0·0015 M)	Na- Lactate. (0·0125 M)	Na- Pyruvate. (0·0125 M)	Na- Succinate. (0·05 M)	Na- Glutamate. (0·05 M)	<i>p</i> - phenylene diamine. (0·05 M)
Allylisopropylbarbituric acid	73	71	67	2	28	0
Phenylethylbarbituric acid	94	79	85	0	50	0
Diethylbarbituric acid	20	22	29	0	0	0
Ethylurethane	17	16	12	0	13	—
Chloretone	93	88	84	0	59	—
Hyoscine	79	73	71	0	60	—
Chloral hydrate	66	90	90	0	62	—
Paraldehyde	3	0	2	0	32	—
Morphine	32	30	30	0	24	—

The inactivity of narcotics towards the oxidation of sodium succinate and *p*-phenylenediamine will be noted. This means that the narcotics can have no injurious effect on oxidations of the brain cell as a whole or on the ability of the cell to activate molecular oxygen. This stands in contrast to the effects of hydrogen cyanide or of carbon monoxide which inhibit the oxidations by the brain of sodium succinate or of *p*-phenylenediamine, or to the action of iodoacetic acid which, whilst inhibiting the oxidation of glucose by brain, does not so markedly affect the oxidation of lactate or pyruvate. The distinctive property of the narcotics is that they inhibit the oxidations by brain of lactate and pyruvate just as powerfully as they inhibit that of glucose. Since there is evidence (Loebel, 1925) that glycolysis of brain is not greatly affected by narcotics at low concentrations and since it appears (Holmes, 1930; Quastel and Wheatley, 1932) that glucose must pass, to a great extent, through lactic acid for its oxidation to occur, it would seem that the primary action of the

narcotics is to affect the activation of lactic and pyruvic acids, their oxidation in this way being inhibited.

The oxidation of glutamic acid is also affected, but, since the velocity of oxidation of this amino acid under normal circumstances is very small, it is unlikely that the effect is of much importance so far as the metabolic changes of the cell, as a whole, are concerned. The main fuel of the brain is lactic acid which is secured from the glucose of the blood (Holmes and Sherif, 1932). It has been shown, too (Quastel and Wheatley, 1932), that the brain appears to make its first demand upon the oxidation of lactic and pyruvic acids, for the presence of these substances brings about a "sparing" of the oxidation of so freely an oxidisable metabolite as succinic acid. In view of these facts, the specific inhibition of the oxidation of lactic and pyruvic acids acquires a special significance. It would seem that as the narcotic is taken up by the brain tissue from the blood stream, it inhibits, at the various nervous centres at which it is adsorbed, the oxidation of glucose or of lactic acid, thereby diminishing the amount of energy available for the functional activity of the cells constituting those centres. The effect would be similar to a condition of anoxæmia obtaining at the nervous centres at which the narcotic is adsorbed—for even though oxygen is freely available, its action upon the essential substrates (glucose or lactic acid) which require oxidation is inhibited.

On comparing the effects of narcotics of different types it will be noted that in each group the more powerfully hypnotic drug has the greater inhibitive action upon the oxidation of glucose by brain. This is shown in Table V, the results referring to guinea-pig brain. Luminal has a greater action than veronal, hyoscine greater than atropine, chloral greater than that of paraldehyde. Paraldehyde, indeed, is almost inert at the concentration used.

Table V.—Percentage Inhibition of extra Oxygen uptake due to Glucose by Narcotics, etc. (0.12 per cent.).

Hyoscine.....	79
Atropine	40
Choral	66
Paraldehyde	3
Luminal	94
Veronal	20
Morphine	32
Strychnine	0
Cocaine	40

It is, of course, useless to make quantitative comparisons between the hypnotic activities and inhibitions of oxidations of substances whose chemical constitutions are widely different. The quantity of morphine, for instance, required to induce a certain degree of narcosis is far less than that of chloretone ; yet for equal concentrations chloretone is a much more powerful inhibitor of the oxidation of glucose by brain. This is probably due to the fact that the particular areas or centres in the nervous system at which morphine is adsorbed are different from those at which chloretone is adsorbed and in view of such specificities of adsorption, quantitative comparisons of differently constituted drugs by the present technique (using whole brain) are valueless. It is clear that the technique must be modified so as to involve the examination of specific areas of the nervous system. Experiments on such lines are now in progress. The results recorded indicate a common mechanism for the action of narcotics and not any particular relationship between hypnotic activity of any narcotic and its influence on the brain as a whole. According to Keller and Fulton (1931) chloral leaves the motor cortex active even when considerably more than the surgical dose is given ; luminal, on the other hand, causes complete abolition of the responses of the motor cortex in full surgical anaesthesia. Yet both chloral and luminal have one property in common—that of inhibiting profoundly the oxidation by whole brain of glucose or lactic and pyruvic acids.

It will now be shown that this property of narcotics applies also to gaseous narcotics.

Action of Gaseous Narcotics.

The gaseous narcotics consisted of :—

- (1) nitrous oxide-air mixture in a ratio 3 : 1 by volume.
- (2) Acetylene-air mixture in a ratio 1 : 1 by volume.
- (3) Air saturated with ether, by being bubbled through ether at room temperature.

In the first two cases, Barcroft vessels containing the brain tissue suspended in the phosphate-saline medium were filled with gaseous narcotics and control vessels were filled with nitrogen-air mixtures of the same composition as the narcotic-air mixtures. The vessels were allowed to shake at 37° for 3 hours, after which the oxygen uptakes of the tissues alone and of the tissue in presence of added glucose solution were measured—the vessels being refilled with the narcotic-air, and nitrogen-air mixtures prior to the measurements being taken.

In the third case, the Barcroft vessels containing tissue were filled with air which had been bubbled through ether at room temperature, and shaken for 3 hours at 37°. The vessels were then filled with air and the oxygen uptakes of the tissue alone and in presence of added glucose were determined. The results (referring to guinea-pig brain) are noted in Table VI.

Table VI.—Inhibitive Action of Gaseous Narcotics.

	N ₂ /air = 3 : 1.	N ₂ O/air 3 : 1.	Inhibition.
			per cent.
O ₂ uptake in c.mm. by tissue alone	219	250	0
Extra O ₂ uptake in presence of glucose (0.05 per cent.)	265	190	28
	N ₂ /air = 1 : 1.	C ₂ H ₂ /air = 1 : 1.	Inhibition.
O ₂ uptake by tissue alone	160	162	0
Extra O ₂ uptake in presence of glucose (0.05 per cent.)	225	152	33
	Air.	Air-ether vapour.	Inhibition.
O ₂ uptake by tissue alone	340	158	53
Extra O ₂ uptake in presence of glucose (0.05 per cent.)	350	173	51

It will be seen that the extra oxygen uptake due to addition of glucose was inhibited 28 per cent. in presence of nitrous oxide, 33 per cent. in presence of acetylene and 51 per cent. in presence of ether vapour. It will be noted also that the oxygen uptakes of the tissues (in absence of added glucose) in the cases of nitrous oxide and acetylene are not less than those in the control vessels where nitrogen was substituted for the narcotic. This is in agreement with the observations of Bülow and Holmes (1932) who state that the oxygen uptake of brain tissue is not affected by the presence of nitrous oxide and acetylene. No account, however, has been taken of the greater solubilities of nitrous oxide and acetylene in lipid material than that of nitrogen. It has been shown (Meyer and Gottlieb-Bilroth, 1920), for instance, that nitrous oxide is 28 times and acetylene 36 times more soluble than nitrogen in oil. The absorption of these gases by the brain lipids would give rise to an apparent oxygen uptake, so that there is no justification for the conclusion, from the figures quoted, that the oxygen uptake of brain is not affected by nitrous oxide or acetylene. This criticism applies also to Bülow and Holmes' conclusion. The determination of the *extra* oxygen uptake due to glucose is not, on the other hand, subject to this criticism and it is clear that the gaseous narcotics inhibit the oxidation of glucose by brain.

Oxidations of Chloroformed Brain.

The effects of narcotics have been demonstrated so far on brain tissue obtained from normal freshly killed animals—the concentrations of narcotics used being in many cases (as with the dialkylbarbituric acid series) of the same order as those required to induce deep narcosis. A more satisfactory quantitative aspect of the effects of the narcotics might be obtained by following the method adopted by Robertson and Stewart (1932) in their experiments on the effects of alcohol on the oxygen uptake of brain tissue, viz., to remove the brain of the animal, whilst narcotised by the drug under investigation, and to examine the oxygen uptake of this tissue in a medium containing the narcotic at a concentration equivalent to that in the blood stream of the animal whilst under narcosis. Experiments carried out in this way with narcotics of the dialkylbarbituric acid series have shown inhibitions of the same order as those quoted already. But owing to the fact that the brain, *as a whole*, is being investigated, only an average effect is obtained and the results can give no true conception of the magnitude of the effect of the drug on some specific area of the brain.

The procedure of examining the oxidative action of normal brain in the presence of *added* narcotic is open to the criticism that the inhibitive effects of narcotics found to occur in this way may not actually occur with the brains of narcotised animals to which narcotics have not been added subsequently.

An attempt to show that such inhibition does occur was carried out successfully using mice as experimental animals and chloroform as the narcotic.

A group of six mice were narcotised by chloroform, killed by severing the carotid and the brains were removed, freed as far as possible from traces of blood, chopped and thoroughly mixed. 0.5 gm. of this brain tissue was examined in the usual way, its oxygen uptake being noted, after 3 hours preliminary oxidation, both alone and in the presence of added glucose. The same procedure was carried out with a group of six mice which were not narcotised. The results are noted in Table VII, where it will be seen that the brains of chloroformed mice showed a 34 per cent. diminution in their ability to oxidise glucose.

Narcotics and Tissue Poisons.

Presumably any substance which diminishes the metabolic activity of a cell may be considered to be a tissue poison. In this sense narcotics are tissue poisons, but usually the phrase "tissue poison" has a general significance implying that the poison affects a number of metabolic changes induced by the tissue. If a characteristic feature, therefore, of a "tissue poison" is a lack of

Table VII.—Oxygen uptakes by Brains of Normal and Chloroformed Mice.

After 3 hours oxidation prior to addition of glucose.		
O ₂ uptake in 2 hours by 0.5 gm. brain of normal mice	c.mm. 210
Extra O ₂ uptake in 2 hours by 0.5 gm. brain of normal mice in presence of glucose (0.05 per cent.)	318
O ₂ uptake in 2 hours by 0.5 gm. brain of chloroformed mice	208
Extra O ₂ uptake in 2 hours by 0.5 gm. brain of chloroformed mice in presence of glucose (0.05 per cent.)	227
Inhibition of oxidation of glucose	per cent. 34

specificity in its inhibitive actions on cell processes, the narcotics, as shown by the experiments quoted above, cannot be regarded as tissue poisons. It is of some interest, however, that certain barbituric acid derivatives are true tissue poisons.

It was found on examining dibrombarbituric acid CBr_2 and

bromisopropylbarbituric acid C_3H_7 that these substances

at low concentrations (0.12 per cent.) were powerful inhibitors of the oxidations of brain. They inhibited the normal oxygen uptake of fresh guinea-pig brain by 65 per cent. and 84 per cent. respectively. Further investigation showed that the inhibitive action of these substances applied not only to the oxidations of glucose and lactate but also to the oxidation of succinate, which was inhibited by over 80 per cent. [It should be pointed out that the intense activity of the dibrom derivative falls off on allowing the aqueous solution to stand, either at room temperature or at 0°, presumably because hydrolysis occurs at the bromine linkages.] It was then found that the oxygen uptake of yeast was inhibited by 25 per cent. by these derivatives—an effect quite contrary to the action of narcotics (see Table II) and there seems, now, no doubt that in contrast to the effects of all dialkylbarbituric acid derivatives studied, these brom-derivatives have non-specific toxic actions, the behaviour with succinate showing the intensity of their injurious effects. The most satisfactory explanation of this behaviour is that they react extremely quickly with tissue proteins or other tissue elements, bringing about damage to the cells with which they come into contact. For this reason, presumably, they

have no narcotic activity when injected intramuscularly into guinea-pigs. They set up an intense local irritant action, showing toxicity, but no narcosis—little or none of the drug actually arriving at the brain after its contact with muscle tissue. When injections via carotid* are made so that the drugs arrive in relatively high concentration at the brain a decided hypnotic effect is obtained.

Mechanism of Narcosis.

It follows, from the data presented, that narcotics have the general property of inhibiting the oxidation by brain of glucose, lactate and pyruvate, whilst leaving certain other oxidations unaffected. Moreover, among narcotics of the same chemical type, those which have the greater hypnotic powers have the greater inhibitive actions.

A simple view of the mechanism of narcosis following from these facts is that the narcotic is adsorbed upon some specific area or centre of the nervous system, this adsorption diminishing the ability of the cells constituting the centre to activate lactic and pyruvic acids. It thus brings about an inhibition of the oxidation of glucose, lactic acid, or pyruvic acid. This inhibition results in a lowered ability of the cell to secure energy to carry out its functional activities. Narcosis—or paralysis of certain specific functional activities—may then ensue. Presumably an equilibrium exists between the quantity of narcotic in the blood and the quantity adsorbed—a reversal being effected as the narcotic is broken down or eliminated by the animal organism. The fact that succinate oxidation is quite unaffected by the narcotics at low concentration is in favour of the view that the tissue suffers little or no irreversible damage by the drugs.

It has been shown by Govodiskay (1925) that there exist chemical differences between functionally different areas of the cortex, these being most marked with regard to the content of cholesterol, cerebroside and saturated phospholipins. It has also been shown by Velluz (1926, *a*, *b*) that the solubility of hypnotics is favoured by the presence of unsaturated linkages in lipoids. In general it was found that the presence of hydroxyl and carboxyl groups and of double bonds in the lipins influenced the solubility of hypnotics. The addition of cholesterol affected partition only in so far as it modified the degree of unsaturation of the lipin.

Thus there is every reason to believe that hypnotics are adsorbed to different extents on different parts of the brain and that their specificity of behaviour

* Kindly carried out by Dr. H. A. Scholberg.

depends ultimately upon such specific adsorption. This, however, will have to be determined by experiment. A point in favour of this view is the fact (Kendle, 1929) that the anæsthetic effect of mixtures of acetylene and ether is an additive function of the constituents. Such additive behaviour is to be anticipated with narcotics whose adsorptions occur at different parts of the nervous system.

It is of some importance to note that the narcotics do not interfere with the activation or access of oxygen to the cell. Their effect, which is upon the mechanism which activates lactic or pyruvic acid, is one which is independent of the presence of oxygen, and anaerobic processes which depend on a similar mechanism for the activation of lactic and pyruvic acids, will also be inhibited by narcotics. It has been found* that anaerobic dehydrogenations due to glucose, lactate and pyruvate are greatly inhibited by narcotics—those of succinate and certain other metabolites are much less affected.

Clearly such a view as has been outlined, helps to reconcile the facts that narcotics inhibit oxidations of the brain and that they also affect anaerobic changes.

The question still remains as to how narcotics bring about a change in the activating power of brain tissue towards lactic and pyruvic acids. It seems possible, in accordance with the suggestion of one of us (Quastel, 1926), that the activation of lactic and pyruvic acids depends upon the preservation of certain electrical conditions at cell surfaces and that the modification of these by the adsorption of narcotics brings about the observed diminution in the power of the cell to activate these acids. It is equally conceivable that the changed rates of activation are due to competition between the narcotics and lactic or pyruvic acid for the enzymes involved.

The writers would like to express their appreciation of the kindness of the firm Hoffmann-La Roche in sending them many dialkylbarbituric acid derivatives, and to the Medical Research Council for a whole-time grant to one of us (A. H. M. W.) and for a grant towards the equipment of this laboratory. They greatly value, too, conversations and correspondence with Dr. M. Guggenheim on the subject of this paper.

Summary.

(1) An investigation has been made of the effects of narcotics on the oxidative processes of the brain.

(2) Narcotics, in general, inhibit the oxidation by brain of glucose, sodium lactate and sodium pyruvate. They also have some inhibiting power on the

* D. R. Davies and J. H. Quastel (1932), 'Biochem. J.' (*in the press*).

oxidation of glutamic acid. They do not inhibit the oxidation by brain of sodium succinate and *p*-phenylenediamine.

(3) The respiration of yeast is unaffected by narcotics at concentrations which are highly active with brain.

(4) Investigation of eight derivatives of the dialkylbarbituric acid series shows that change in the hypnotic activity of these derivatives is accompanied by a similar change in their inhibitive powers.

(5) Investigation of different narcotics shows that among narcotics belonging to the same chemical type, those with the greater hypnotic activity have the greater inhibitive action on brain oxidation.

(6) The brains of chloroformed mice show a diminished activity to oxidise glucose.

(7) It is suggested that a view of the mechanism of narcosis which fits most closely to the facts, is that the narcotic is adsorbed from the blood at a specific area or centre of the nervous system. Here it brings about a diminution of the ability of the cells constituting the nervous centre to activate lactic or pyruvic acid, in this way inhibiting the oxidation by these cells of glucose, lactic and pyruvic acids. The access or activation of oxygen is quite unimpaired, but the diminished ability of the cells to oxidise glucose or lactic acid results in a lowering of the amount of energy available for the cells to accomplish their functional activities. Narcosis—or a depression of the normal functional activity of the nervous centre in question—may then ensue.

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A Genetical Formula for the Inheritance of Intelligence in Man.

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The inheritance of natural mental ability was first demonstrated statistically by Galton (1869) and his conclusions have since been considerably amplified and developed by Pearson (1904, 1919) and Woods (1906). So far no attempt has been made to express the problem in terms of a genetical formula, but recent progress in experimental psychology and genetics has made this possible.

Material.

Two distinct sets of material have been used in this investigation.

(1) The cumulative results of a series of studies of the natural mental ability of the parents and offspring of 194 Leicestershire families continued through many years, which involved the personal investigation and gradings for intelligence of 388 parents and their 812 offspring, most of whom were recorded as adults. The same families were studied for the determination of the inheritance of Eye-colour in Man (Hurst, 1908, 1925). The material includes representative families of various classes and conditions of life in a rural manufacturing and agricultural district of South-west Leicestershire and provides a fair sample of the general population of the area, the only conscious selection exercised being large families of faithful matrons containing the highest and lowest grades of intelligence in the district. This material is distinguished as the L.F. data (Leicestershire families).

(2) The independent statistical material presented by Woods (1906) on the "intellect" gradings of the leading Royal Families of Europe has been utilised in order to increase the range of observations in grades, space and time. Woods' data include the whole adult issue of 212 families in England, Germany, France, the Netherlands, Spain, Portugal, Austria, Italy, Russia, Denmark and Sweden. The period covered extends back to the sixteenth century except in the case of Spain and Portugal which go back to the eleventh century. This material involved the independent grading for "intellect" of 424 parents and their 558 offspring and is distinguished as the R.F. data (Royal Families).

It is difficult to conceive two sets of material more widely different and so suitable for constructing a general genetical formula applicable to a wide range

of families. The L.F. data represent a relatively constant and narrow range of heredity associated with a variable social environment with extremes of poverty and wealth, while the R.F. data represent an inconstant and extremely wide range of heredity associated with a more uniform social environment. Further, the one is typically English and modern while the other includes families of 11 different nationalities and is spread over a period of several centuries.

Altogether the total material available for study includes 406 families involving 812 parents and their 1370 offspring.

Definitions and Methods.

Throughout the investigation the concept "intelligence" is taken to represent natural mental ability as expressed in general mental activities and achievements, on the lines and definitions laid down by Galton (1869), Woods (1906), and Spearman (1927). The concept corresponds with Galton's "natural ability," Woods' "intellect," and Spearman's "general factor g ."*

The statistical data of Galton (1869) and Woods (1906) show that general intelligence in adults grades continuously from high to low both in general and in selected populations. In order therefore to distinguish and define the different degrees of intelligence, it is necessary to divide them into grades of equal value. Galton (1869) divides "natural ability" into 14 or more grades which Woods (1906) reduces to 10. In this investigation, since Woods' data are used as they stand, it is necessary to adopt his grading of "intellect" throughout, both for the R.F. and the L.F. data. As in all other attempts to grade "intelligence" both in children and adults, the gradings are naturally imperfect and in a few cases they are probably erroneous through pathological complications; but, taken as a whole, the results show a general agreement,

* By the criterion of "tetrad differences" based on the correlation coefficients of mental tests, Spearman has demonstrated the existence of a statistical unit-factor " g " for general intelligence or "mental energy," which is closely associated with, but demonstrably distinct from the secondary factor " s " which represents "special abilities" or specific aptitudes of various kinds. Spearman's tetrad equation, $r_{ap} r_{bq} - r_{aq} r_{bp} = 0$ demonstrating " g ," has been shown mathematically to apply for all manner of distributions and any number of variables. The psychological discovery of a perfectly definite quantitative value " g ," establishes the earlier assumptions of Galton (1869) and Woods (1906) and clears the way for a genetical investigation of the problem of the inheritance of intelligence in Man. Tests show that Spearman's " g " is constant for any normal individual from the age of 13-16 years up to the onset of senility, while it is greatly variable in degree or power in different individuals.

which, with the constant ratios found in the two distinct sets of data, signifies a close approximation to reality. On the whole, so far as performance can be regarded as a test or measure of capacity, the gradings of Galton and Woods are probably the best that can be devised for adults.

Grades of Intelligence.

On this basis there are 10 grades of intelligence from the highest grade 10 to the lowest grade 1, as defined and characterised below.

Grade 10. Illustrious.—The highest grade of intelligence found in the data, amounting to illustrious genius of historical rank; the grade X of Galton and grade 10 of Woods. Individuals of this grade are extremely rare in ordinary populations, and Galton estimates a total number of 400 individuals of this grade in the whole of historical time.

Grade 9. Eminent.—Individuals of this grade may be highly eminent but not illustrious. They have distinguished themselves frequently by their original work, or have proved themselves to be national or international leaders. The grade G of Galton and grade 9 of Woods.

Grade 8. Brilliant.—Individuals with brilliant intellects but not eminent, distinguished by their originality and versatility. The grade F of Galton and grade 8 of Woods.

Grade 7. Talented.—Individuals who are talented but not brilliant, distinguished by original ability in narrow fields, but not versatile. The grade E of Galton and grade 7 of Woods.

Grade 6. Able.—Superior individuals with more than average intelligence, who obtain the ordinary prizes of life, but are not gifted with original talents. The grade D of Galton and grade 6 of Woods.

Grade 5. Mediocre.—Average individuals, neither superior nor inferior in intelligence, including the great majority of an ordinary population. The mediocre grades of Galton and grade 5 of Woods, including also his negative grade "obscure," comprising individuals not sufficiently noteworthy to be graded as superior or inferior in intelligence. In the L.F. data these are carefully and definitely distinguished from grades 4 and 6, but in the R.F. data, judging by their frequencies as compared with those of L.F., it is highly probable that some of those graded as "obscure" belong either to grade 6 or 4, but to no higher or lower grade. In the absence of definite evidence, however, it is considered better to retain these in the mediocre grade 5, but distinguished

by the letter M rather than to attempt an arbitrary proportionate allotment to adjacent grades.

Grade 4. Dull.—Inferior individuals, below the typical average in intelligence, varying from merely simple-minded to dull and stupid. The grade *d* of Galton and grade 4 of Woods.

Grade 3. Subnormal.—Weak-minded, puerile, incompetent and mentally dependent. The grade *e* of Galton and grade 3 of Woods.

Grade 2. Moron.—Mentally defective, unstable, lacking judgment and control, requiring constant supervision. The grade *f* of Galton and grade 2 of Woods.

Grade 1. Imbecile.—Mentally helpless and physically dependent. The grade *g* of Galton and grade 1 of Woods.

In the Leicestershire families the gradings were made on the basis of the above definitions and characterisations, and all were graded by their general mental output and achievements, ascertained through an intimate personal knowledge of their mental activities through many years of observation. As a rule little difficulty was found in assigning an adult to the recorded grade, but any dubious borderline cases were placed in the lower grade. A few cases were found in which old or young persons decreased or increased their grade during the period of investigation; in such cases the highest grade attained was recorded. Those who died young, if recorded, were placed in their optimum grade. A few children are included in the L.F. data, and these, with the low-grade adults, were tested for I.Q. by the Stanford-Binet and the Healy Pictorial Intelligence tests and assigned to the adult gradings on the basis of 20 juvenile I.Q. to each adult grade, as follows :—20 I.Q. = Grade 1 (Imbecile). 40 I.Q. = Grade 2 (Moron). 60 I.Q. = Grade 3 (Subnormal). 80 I.Q. = Grade 4 (Dull). 100 I.Q. = Grade 5 (Mediocre). 120 I.Q. = Grade 6 (Able). 140 I.Q. = Grade 7 (Talented). 160 I.Q. = Grade 8 (Brilliant). 180 I.Q. = Grade 9 (Eminent). 200 I.Q. = Grade 10 (Illustrious).

Table I presents a summary of the frequencies of the 10 grades of intelligence found in the parents and offspring of the two sets of data, in the Leicestershire families and the Royal families respectively. The close correspondence in frequency between parents and offspring of each grade, in each set of data, is marked and significant.

The two sets of data, however, show considerable differences. On the whole the Royal families have a much larger proportion of high and low grades of intelligence, while the Leicestershire families have an excess of the mediocre grade. The 22·3 per cent. excess of grade 5 in the L.F. data is remarkable

and genetically significant. Genetical analyses of the grade 5 matings in the L.F. data reveal the existence of two distinct types of families, (*a*) and (*b*), which involve corresponding genetical differences in grade 5 parents. (*a*) is a non-segregating type of family which consists of grade 5 offspring only (Tables II and IV), while (*b*) is a segregating type of family which consists of offspring of various grades (Tables III, V and VI). Since type (*a*) family can be tested genetically in families of large size, the families are arranged in the tables in order of their size. In order to compare the two sets of material, the R.F. data have been tabulated on the same lines as the L.F. data. Tables VII and IX give details of each of the (*a*) families, and Tables VIII, X and XI the (*b*) families. Tables VII and VIII represent the matings of grade 5 *inter se* (including M), Tables IX and X the matings of grade 5 (including M) with other grades, and Table XI the matings *inter se* of grades other than 5 (or M).

Discussion.

Table I shows that 70·8 per cent. of the parents in the L.F. data are of mediocre intelligence (grade 5). The evidence in Tables II and III, that these grade 5 parents regularly produce two distinct family types (*a*) non-segregating

DATA.

Table 1.—Frequencies of Intelligence Grades in Parents and Offspring.

Grades :	10.	9.	8.	7.	6.	5.	4.	3.	2.	1.	Totals.
Parents, L.F. data	0	0	2	8	57	275	41	5	0	0	388
Per cent.	0·0	0·0	0·5	2·0	14·6	70·8	10·5	1·2	0·0	0·0	
Offspring, L.F. data	0	0	4	15	115	590	71	13	2	2	812
Per cent.	0·0	0·0	0·4	1·8	14·1	72·6	8·7	1·6	0·2	0·2	
Totals	0	0	6	23	172	865	112	18	2	2	1200
Per cent.	0·0	0·0	0·5	1·9	14·3	72·0	9·3	1·5	0·1	0·1	

Parents, R.F. data	10	11	32	44	43	221	28	21	12	2	424
Per cent.	2·3	2·5	7·5	10·3	10·1	52·1	6·6	4·9	2·8	0·4	
Offspring, R.F. data	8	22	30	57	65	268	44	33	22	9	558
Per cent.	1·4	3·9	5·3	10·2	11·6	48·0	7·8	5·9	3·9	1·6	
Totals	18	33	62	101	108	489	72	54	34	11	982
Per cent.	1·8	3·3	6·3	10·2	10·9	49·7	7·3	5·4	3·4	1·1	

Table II.—(a) Non-segregating Families (L.F. Data).

Grade 5 × Grade 5.

Number in family.	Family identity numbers.	Number of families.	Total offspring all grade 5.
12	122—124	2	24
11	99	1	11
10	94—141	2	20
9	98—130	2	18
8	125	1	8
7	92—93—105—112—140	5	35
6	63—81—95—128—142	5	30
5	60—78—100—107—117—132	6	30
4	45—59—96—111—113—119—138	7	28
3	52—74—77—90—101—108—118—135	8	24
2	49—50—62—72—73—75—86—109—110	9	18
1	43—46—47—64—65—68—84—85—116	9	9
		57	255

and (b) segregating, indicates the inheritance of intelligence on a genetical basis. On this basis, one (or both) of the parents of the large non-segregating families is homozygous for a pair of dominant genes *NN* which determine normal mediocre intelligence, while both parents of the segregating families are carrying a recessive gene *n*, which coming together (*nn*) cause offspring of abnormal grades of intelligence to appear. Thus mediocre parents of grade 5 may be of three different genic constitutions, *NN*, *Nn* or *nn*, while their offspring may be either all mediocre or of various grades. In the presence of the dominant gene *N* (*NN* or *Nn*) an individual is of normal mediocre intelligence, while in the presence of *nn* an individual may be of any grade of intelligence (grade 10 to grade 1). Confirmation of this has been found in the possibility of assigning consistent individual genic constitutions to a large number of the parents in the L.F. and R.F. data by the method of genetical fitting of parents with offspring, which in human genetics is equivalent to the experimental tests in plants and animals. In this way it has been found that a large majority of the parents in the L.F. data are *NN* or *Nn*, while a majority of the parents in the R.F. data are *nn* in constitution. This finding is in agreement with the evidence in Table I that 72·6 per cent. of the offspring in the L.F. data are of grade 5 while in the R.F. data only 48·0 per cent. are of this grade. The remarkable differences between the Royal families of Europe and the Leicestershire families in their grades of intelligence can therefore be largely,

Table III.—(b) Segregating Families (L.F. Data).

Grade 5 \times Grade 5.

Number in family.	Grades of offspring.										Identity family number.
	10.	9.	8.	7.	6.	5.	4.	3.	2.	1.	
9	2	7	80
9	2	6	1	.	.	.	134
8	4	4	54
8	6	1	0	1	.	58
8	6	2	.	.	.	104
7	5	2	.	.	.	79
6	1	5	67
6	1	4	1	.	.	.	70
6	1	3	2	.	.	.	88
6	2	4	97
6	3	2	0	1	.	.	126
6	.	.	.	1	1	2	2	.	.	.	129
6	1	5	137
6	1	5	139
6	1	5	143
5	.	.	1	0	1	3	55
5	3	2	.	.	.	102
5	2	2	1	.	.	.	103
5	1	4	127
4	1	2	1	.	.	.	44
4	1	3	115
4	1	3	120
4	1	3	123
4	1	2	1	.	.	.	133
3	.	.	.	2	0	1	42
3	2	1	.	.	.	48
3	1	2	53
3	1	1	0	1	.	.	61
3	2	0	1	.	.	82
3	2	1	.	.	.	89
3	2	1	.	.	.	91
3	2	1	.	.	.	114
3	1	2	136
2	1	1	51
2	.	.	.	1	0	1	57
2	2	.	.	69
2	1	1	.	.	.	71
2	1	1	.	.	.	83
2	1	0	1	.	.	106
2	1	1	.	.	.	131
1	.	.	.	1	56
1	1	66
1	1	76
1	1	87
1	1	.	.	.	121
189	0	0	1	5	36	116	24	6	1	0	45*

Number of families.

Table IV.—(b) Non-segregating Families (L.F. Data).

Grade 5 \times Other Grades.

Number in family.	Parental grades.	Family identity numbers.	Number of families.	Total offspring all grade 5.
6	5 \times 6	170—172	2	12
6	5 \times 4	7—11—13	3	18
5	5 \times 6	165	1	5
4	5 \times 4	28	1	4
3	5 \times 6	161—166—178	3	9
3	5 \times 4	10—18—29—31	4	12
2	5 \times 6	147—155	2	4
2	5 \times 4	24—30—33	3	6
1	5 \times 4	25	1	1
			20	71

if not wholly, expressed in terms of the frequency of the genes n and N in the respective populations.

That the Royal families investigated have not always carried a preponderance of n genes is evident from a study of their early history. Woods (1906) has analysed the records of the ancestral families of Mecklenburg (A.D. 960–1860), Montmorency (1100–1567), Condé (1300–1569), Hohenzollern (1100–1660), and Oldenburg (1588–1863) and finds that, measured by the standards used for the R.F. data, only 12 individuals of the higher grades of intelligence can be traced during these periods, in the numerous families of the direct lines and their male and female collateral ascendants to the third generation. It is evident that throughout this early period, extending over several centuries, these ancestral families of the modern Royal families carried a majority of N genes, as in the Leicestershire families and other normal populations. The high grade founders of the modern Royal families were nn and the frequent intermarriages recorded in the R.F. data between Royal families carrying the n gene mutation have naturally resulted in an increase of n genes, and have given rise to the larger numbers of high and low grades of intelligence found in the R.F. data. It will be noted that the increase of n genes in the Royal families has involved an increase of both high and low grades of intelligence.

High and Low Grades.—The genetical problem of the inheritance of the recessive high and low grades of intelligence (nn) is more complex than that of the dominant normal mediocre grade of the NN and Nn parents. In the segregating families of the “selfings” and “backcrossings” of grade 5 in

Table V.—(b) Segregating Families (L.F. Data).
Grade 5 × Other Grades.

Number in family.	Parental grades.	Grades of offspring.										Identity family number.
		10.	9.	8.	7.	6.	5.	4.	3.	2.	1.	
13	5 × 4	5	5	3	.	.	.	20
9	5 × 4	3	3	2	1	.	.	16
9	5 × 4	7	2	.	.	.	19
8	5 × 4	1	4	3	.	.	.	27
8	5 × 6	.	.	.	1	1	6	179
7	5 × 4	5	2	.	.	.	8
7	5 × 4	4	2	1	.	.	21
6	5 × 4	4	2	.	.	.	32
6	5 × 6	2	4	169
6	5 × 7	.	.	.	1	1	4	183
5	5 × 4	3	2	.	.	.	17
5	5 × 6	2	3	145
5	5 × 6	2	3	154
5	5 × 6	.	.	.	1	0	3	1	.	.	.	157
5	5 × 6	2	3	162
5	5 × 6	3	2	173
5	5 × 6	2	3	174
5	5 × 6	1	3	1	.	.	.	175
4	5 × 6	.	.	.	1	0	2	1	.	.	.	150
4	5 × 6	1	2	1	.	.	.	151
4	5 × 6	2	2	152
4	5 × 6	1	3	156
4	5 × 6	2	2	167
4	5 × 6	1	3	180
4	5 × 4	2	2	.	.	.	12
4	5 × 4	1	3	23
4	5 × 7	2	2	185
3	5 × 6	.	.	1	0	2	149
3	5 × 6	1	2	158
3	5 × 6	1	2	160
3	5 × 6	1	1	1	.	.	.	163
3	5 × 6	2	1	171
3	5 × 6	1	2	178
3	5 × 4	2	1	.	.	.	9
3	5 × 4	2	1	.	.	.	22
3	5 × 4	1	1	1	.	.	.	26
3	5 × 7	.	.	.	1	1	1	184
3	5 × 3	2	1	5
2	5 × 8	1	1	.	194
2	5 × 6	1	1	146
2	5 × 6	1	1	153
2	5 × 6	1	1	.	.	.	159
2	5 × 6	1	1	164
2	5 × 6	1	1	168
2	5 × 7	.	.	1	0	1	181
2	5 × 7	.	.	.	1	1	182
2	5 × 3	1	0	1	.	.	.	6
1	5 × 6	1	148
1	5 × 6	1	176
1	5 × 4	1	.	.	14
1	5 × 3	1	4
210		0	0	2	6	57	110	30	4	1	0	51*

* Number of families.

Table VI.—(b) Segregating Families (L.F. Data).
High and Low Grade Matings (excluding Grade 5).

Number in family.	Parental grades.	Grades of offspring.										Identity family number.
		10.	9.	8.	7.	6.	5.	4.	3.	2.	1.	
7	6 × 4	1	4	2	.	.	.	38
6	6 × 4	1	5	39
6	6 × 6	2	3	1	.	.	.	190
6	6 × 6	3	3	187
6	7 × 6	.	.	.	2	2	2	192
6	7 × 4	3	2	1	.	.	.	144
5	4 × 4	2	3	.	.	.	2
5	6 × 4	3	2	.	.	.	34
5	6 × 4	2	3	35
5	6 × 6	1	4	186
4	3 × 3	2	0	2	1
4	4 × 4	4	.	.	.	15
4	6 × 6	2	2	191
4	6 × 6	.	.	.	2	2	188
3	6 × 4	1	2	37
3	6 × 6	1	0	1	1	.	.	189
2	4 × 4	2	.	.	.	3
2	6 × 4	1	1	.	.	.	41
2	6 × 4	1	1	40
1	6 × 4	1	36
1	8 × 7	.	.	1	193
87		0	0	1	4	22	38	17	3	0	2	21*

* Number of families.

Table VII.—(a) Non-segregating Families (R.F. Data).
Grade 5 × Grade 5 (including M).

Number in family.	Parental grades.	Family identity numbers.	Number of families.	Offspring all M or grade 5.	
				M.	5.
7	M × M	852	1	7	.
5	5 × M	714	1	4	1
2	5 × M	298	1	1	1
1	5 × M	834—291	2	1	1
1	M × M	835—836—838—840—841—842—843 ..	7	7	.
1	M × M	844—845—847—848—137—75—139 ..	7	6	1
1	M × M	218—856—857—858—860—592—864 ..	7	5	2
1	M × M	865—866	2	2	.
			28	33	6
			Total ..	39	

Table VIII.—(b) Segregating Families (R.F. Data).

Grade 5 \times Grade 5 (including M).

Number in family.	Parental grades.	Grades of offspring.											Identity family number.
		10.	9.	8.	7.	6.	5.	M.	4.	3.	2.	1.	
12	5 \times 5	.	.	.	1	1	3	1	4	2	.	.	18
11	5 \times 5	.	.	.	2	1	2	5	0	1	.	.	515
10	M \times M	.	.	1	0	0	0	9	850
9	5 \times M	1	4	2	2	.	.	347
8	5 \times M	0	7	299
6	5 \times M	.	.	.	1	1	1	1	0	1	1	.	703
6	M \times M	.	1	0	0	0	0	5	853
5	5 \times 5	.	.	.	2	0	2	0	0	1	.	.	390
5	M \times M	.	1	0	0	0	0	4	854
3	5 \times M	2	1	185
2	5 \times 5	1	1	149
2	5 \times M	1	1	130
2	5 \times M	.	.	.	1	0	1	366
2	5 \times M	.	.	.	1	0	0	1	592
2	M \times M	.	1	0	0	0	0	1	855
1	5 \times 5	1	833
1	5 \times M	1	62
1	5 \times M	1	115
1	5 \times M	1	.	.	.	43
1	5 \times M	1	360
1	5 \times M	1	219
1	M \times M	.	.	1	837
1	M \times M	1	839
1	M \times M	.	.	.	1	846
1	M \times M	.	.	.	1	849
1	M \times M	.	1	851
1	M \times M	.	.	.	1	859
1	M \times M	1	861
1	M \times M	1	862
1	M \times M	.	.	1	863
1	M \times M	.	.	1	867
1	M \times M	.	1	868
102		1	5	4	11	15	13	38	7	7	1	0	32*

* Number of families.

Tables III, V, VIII and X, the frequent approximations to Mendelian ratios in adjacent grades and the frequent discontinuity of occurrence of the 10 grades point to a genetical basis of considerable complexity. The crucial evidence of the recessive matings ($nn \times nn$) in Tables VI and XI, from which by hypothesis all NN and Nn parents are excluded, is, however, peculiarly significant in its relatively smooth frequency curve, this and the constant and considerable decrease of frequency of the higher and lower grades as their distance from the middle grade 5 increases, in the other segregating families, definitely point to the co-operative action of five pairs of multiple modifying

Table IX.—(a) Non-segregating Families (R.F. Data).
Grade 5 (including M) \times Other Grades.

Number in family.	Parental grades.	Family identity numbers.	Number of families.	Offspring all M or grade 5.	
				M.	5.
6	M \times 7	177	1	4	2
3	M \times 7	316	1	3	.
3	M \times 8	364	1	3	.
2	M \times 4	215	1	2	.
2	M \times 7	60	1	1	1
2	M \times 9	269	1	2	.
2	M \times 10	258 _a	1	2	.
1	M \times 4	872	1	.	1
1	5 \times 4	869	1	.	1
1	5 \times 7	870	1	1	.
1	M \times 7	90—884—603	3	3	.
1	M \times 3	388—561 _a	2	.	2
1	M \times 6	874—116—880	3	2	1
1	M \times 8	514	1	.	1
			19	23	9
			Total ...	32	

Table X.—(b) Segregating Families (R.F. Data).
Grade 5 (including M) \times Other Grades.

Number in family.	Parental grades.	Grades of offspring.											Identity family number.
		10.	9.	8.	7.	6.	5.	M.	4.	3.	2.	1.	
14	M \times 6	.	.	.	1	2	1	10	259
10	5 \times 7	.	.	1	2	0	1	3	2	1	.	.	390
9	5 \times 6	.	.	.	1	0	0	8	592
7	M \times 6	.	.	.	2	3	0	2	414
7	M \times 8	.	.	1	0	0	0	6	322
6	5 \times 3	.	.	.	1	1	2	0	2	.	.	.	727
6	5 \times 8	1	1	1	1	0	2	.	202
6	M \times 8	.	.	1	1	0	2	0	1	1	.	.	403
6	M \times 10	.	.	.	1	0	0	5	258 _c
5	5 \times 9	.	.	1	0	1	1	0	0	2	.	.	372
5	M \times 1	1	0	3	0	0	0	1	901
5	M \times 2	.	.	.	2	0	2	0	1	.	.	.	526
5	M \times 8	.	.	1	0	1	0	3	277
4	5 \times 4	1	0	3	730
4	M \times 6	1	1	1	0	0	0	1	875
4	M \times 6	2	1	1	537
4	M \times 7	1	1	2	881
4	M \times 7	.	.	1	0	0	1	2	345
4	M \times 7	.	2	0	0	0	0	0	0	0	0	2	745
4	M \times 8	.	1	0	0	1	0	2	324
4	M \times 8	.	.	1	0	0	0	3	335
3	5 \times 2	1	0	0	0	0	2	.	381
3	5 \times 3	1	0	2	528

Table X—(continued).

Number in family.	Parental grades.	Grades of offspring.											Identity family number.
		10.	9.	8.	7.	6.	5.	M.	4.	3.	2.	1.	
3	5 × 4	2	0	1	412
3	5 × 4	1	0	.	0	2	.	715
3	5 × 6	1	1	0	1	.	.	611
3	M × 3	1	0	0	0	0	0	2	377
3	M × 7	.	.	.	2	0	0	1	314
3	M × 7	.	.	.	1	0	0	2	178
3	M × 7	.	.	.	1	0	0	1	1	.	.	.	627
3	M × 9	.	.	1	1	0	0	1	658
2	5 × 4	.	.	1	0	0	0	1	344
2	5 × 6	2	.	528
2	5 × 6	2	.	537
2	5 × 6	.	.	.	1	0	0	0	1	.	.	.	667
2	M × 1	1	.	.	751
2	M × 4	1	0	1	.	.	310
2	M × 6	1	1	.	.	.	302
2	M × 7	0	1	359
2	M × 7	.	.	.	1	883
2	M × 7	.	.	.	1	0	0	1	682
2	M × 8	1	1	80
2	M × 8	.	.	.	1	0	0	1	336
2	M × 9	.	.	.	1	0	0	0	0	1	.	.	753 _b
2	M × 10	.	1	0	0	0	0	1	258 _b
1	5 × 3	1	539
1	5 × 4	1	.	518
1	5 × 4	1	.	.	.	411
1	5 × 6	.	.	.	1	54
1	5 × 6	1	185
1	5 × 8	.	.	1	73
1	5 × 8	1	.	.	.	616
1	M × 3	.	.	.	1	351
1	M × 3	1	.	.	546
1	M × 3	1	561 _b
1	M × 4	1	.	.	.	871
1	M × 4	1	873
1	M × 6	1	876
1	M × 6	1	.	.	.	877
1	M × 6	1	72
1	M × 6	1	82
1	M × 6	1	.	.	.	878
1	M × 6	1	879
1	M × 6	.	.	.	1	743
1	M × 7	1	.	.	.	583
1	M × 7	1	.	.	.	885
1	M × 7	.	.	.	1	886
1	M × 7	1	662
1	M × 7	.	.	.	1	882
1	M × 7	1	684
1	M × 8	.	1	278
1	M × 8	.	.	.	1	887
1	M × 8	1	.	.	.	619
1	M × 8	.	.	1	888
1	M × 8	1	889
1	M × 8	1	890
1	M × 8	1	742
1	M × 9	.	.	.	1	891
1	M × 9	.	1	892
1	M × 9	1	.	753 _a
1	M × 10	.	.	.	1	343
1	M × 10	.	1	893
918		3	8	12	31	28	16	69	19	12	13	7	82*

Table XI.—(b) Segregating Families (R.F. Data).
High and Low Grade Matings (excluding Grade 5 and M).

Number in family.	Parental grades.	Grades of offspring.											Identity family number.
		10.	9.	8.	7.	6.	5.	M.	4.	3.	2.	1.	
12	6 × 2	.	1	0	0	4	2	2	2	1	.	.	634
11	9 × 4	.	.	2	2	3	2	1	1	.	.	.	627
10	7 × 7	2	2	1	1	1	2	1	227
8	7 × 6	2	4	0	1	0	1	.	583
8	8 × 3	.	1	2	2	0	0	3	192
7	6 × 3	2	2	1	0	1	1	.	10
7	8 × 3	2	0	0	4	1	.	.	8
6	4 × 3	3	2	1	.	.	.	388
6	4 × 4	1	2	1	0	1	1	640
6	7 × 7	.	.	.	2	0	0	4	173
6	10 × 6	.	1	1	1	0	2	1	688
6	10 × 7	.	.	1	0	0	0	4	1	.	.	.	1
5	6 × 3	1	0	1	0	1	2	.	545
4	7 × 2	1	0	1	1	1	.	.	386
4	7 × 4	.	.	.	1	1	1	0	1	.	.	.	48
4	7 × 6	2	0	2	603
4	8 × 6	1	2	1	514
3	6 × 3	.	.	1	1	0	0	1	312
3	7 × 6	2	0	0	0	0	0	0	1	.	.	.	341
3	7 × 6	.	1	1	0	0	1	450
2	3 × 2	.	.	1	0	0	1	511
2	4 × 3	.	.	.	1	0	0	0	0	1	.	.	374
2	4 × 4	2	894
2	6 × 2	1	0	0	1	.	.	386
2	6 × 2	1	0	1	.	.	.	551
2	6 × 2	1	0	0	0	1	.	.	554
2	6 × 2	1	0	1	.	.	764
2	6 × 3	1	1	.	.	552
2	6 × 4	.	.	.	1	0	0	0	0	1	.	.	2
2	6 × 4	1	1	.	.	.	234
2	7 × 2	1	1	.	.	.	546
2	10 × 8	1	0	0	1	.	429
2	9 × 4	1	0	0	0	0	1	711
1	4 × 2	1	.	.	722
1	4 × 3	1	380
1	6 × 4	.	.	.	1	27
1	6 × 4	.	1	622
1	7 × 2	1	.	379
1	7 × 3	.	.	1	380
1	7 × 4	1	.	895
1	7 × 7	.	1	896
1	8 × 3	.	.	.	1	226
1	8 × 6	.	.	1	897
1	8 × 7	1	898
1	8 × 7	1	899
1	8 × 8	.	1	657
1	8 × 8	.	.	1	900
1	9 × 7	1	.	.	510
1	9 × 8	.	.	.	1	675
1	10 × 6	1	.	.	224
1	10 × 7	.	.	1	258 ^d
167		4	9	14	15	22	28	33	18	14	8	2	51*

* Number of families.

genes, which are equal in their cumulative action, as in the modifying genes for broad grains and glumes of the "Marquis" wheat (Philipstchenko, 1927)* and the modifying genes concerned in the inheritance of musical ability in man (Hurst 1932).

Genetical Formula.

On the above analysis of the data a basic genetical formula for the inheritance of natural intelligence in man has been provisionally constructed. It is a hexagenic formula of the order of 6 pairs of genes based on 1 pair of major genes Nn , representing normal and abnormal intelligence, plus 5 pairs of minor genes Aa , Bb , Cc , Dd , Ee , which act as modifiers with the major genes nn . On the present evidence the minor genes appear to act quantitatively and are equal and cumulative in their effect, so that the addition of any single dominant gene of the 5, increases the effect to the extent of a grade, while the subtraction of a dominant gene and the substitution of a recessive gene decreases the effect by a grade. Thus the highest grade 10 (Illustrious) is in minor genic constitution $AABBCCDDEE$ with 10 dominant increasers and no recessive decreaseers, grade 9 (Eminent) has 9 increasers and 1 decreaseer and so on to grade 1 (Imbecile) with 1 increaser and 9 decreaseers, while grade 5 (Mediocre) of the abnormal type (nn) is equally balanced with 5 dominant increasers and 5 recessive decreaseers, *e.g.*, $AaBbCcDdEe$. It will be noted that in this scheme an 11th grade is possible, grade 0, with the genic constitution $aabbccdddee$, *i.e.*, with 10 recessive decreaseers and no dominant increasers. This grade is not found in the L.F. data and is not recorded in the R.F. data, but it may be taken to represent the lowest grade of idiocy, which is rarely recorded except in medical literature; it is semi-lethal, seldom surviving to an adult stage.

It is important to note that these 5 pairs of minor genes $Aa \dots Ee$ which determine the high and low grades of intelligence act only in the presence of the major recessive genes nn . In the presence of the major dominant genes NN and Nn the action of all the minor genes $AaBbCcDdEe$ is suspended and consequently the appearance of high and low grades of intelligence is inhibited. It is possible, therefore, for a mediocre individual with NN genes to be carrying also the 10 increaser genes $A \dots E$ of a genius or the 10 decreaseer genes $a \dots e$ of an idiot or any combination of these. Experimental parallels of modifying genes of this nature in animals and plants are found in the 8 modifiers

* For a translation of this important paper on the "Absorbing Influence of Crossing" I am indebted to Sir Rowland Biffen, F.R.S., and Dr. Hudson, of the Imperial Bureau of Plant Genetics, Cambridge.

of recessive eosin eye-colour in *Drosophila* (Bridges, 1919), which apparently have no effect on dominant red eye-colour, and in the 5 modifiers of recessive broad grains and glumes of wheat (Philipschenko, 1927), which apparently have no effect on the dominant narrow grains and glumes.

In one respect, however, the human data and formula presented do not appear to be precisely parallel with the cases cited above. In both these cases the dominant major character appears to represent the end of a series exhibited by the recessive character in combination with modifiers. In the case of human intelligence, on the other hand, the dominant major character seems to represent the middle of the series exhibited by the recessive character in combination with modifiers. In view of this an objection might be raised on the ground that the relations between the dominant and recessive characters in this case are unusual. The difficulty is removed, however, when it is realised that the major Mendelian characters in this case are not really dominant grade 5 contrasting with recessive grades 0 . . . 5 . . . 10, but rather dominant normal stable intelligence contrasting with recessive abnormal variable intelligence. So far it has not been possible to distinguish phenotypically between the NN mediocres of grade 5 and the *nn* mediocres of the same grade. The evidence available in the data suggests that the numerous NN individuals of grade 5 tend to be more uniform and less variable than the relatively rare *nn* individuals of grade 5, but the numbers of the latter are insufficient to determine this point. Whether the two types of grade 5 can be distinguished phenotypically or not, there is no doubt that they are genotypically distinct since they produce different kinds of offspring (*cf.* Tables II and III, IV and V, VII and VIII and IX and X).

The present data relate only to the White European races and since there are 729 possible genotypes in this hexagenic scheme, the genetical formula can be expressed in 729 different ways, but its typical expression may be taken as that of its maximum heterozygosity from which all the other expressions follow as genetical consequences. Future research among various races will, no doubt, modify this basic and provisional formula by the addition, subtraction or substitution of genes, but on the present evidence it may be set out as follows, as a temporary working hypothesis, realising that as a scientific formula eventual modifications are inevitable

$$[Nn + (Aa + Bb + Cc + Dd + Ee)]$$

where N and *n* are a major pair of genes for normal and abnormal intelligence and A and *a* . . . E and *e* are minor pairs of modifying genes. In the presence

of N , $Aa \dots Ee$ have no effect and the phenotype is grade 5 (mediocre intelligence). In the presence of m , $A \dots E$ act as equal and cumulative increasers of intelligence from the lowest grade 1 to the highest grade 10, while $a \dots e$ act as decreasers.

Formula Tests.

The next step is to test the formula as a working hypothesis on various families and populations. Naturally, in the case of man, direct experimental tests are not available, and if they were it would be difficult to carry them out owing to the length of time required to obtain several generations of adults. Corresponding tests, which are genetically equivalent, can, however, be applied forthwith. The most simple and probably the most useful of these is that of testing the fitness of the formula to the observed results of the matings of the different grades with one another in various families and populations. Expectation tables based on the formula have been prepared on these lines and qualitatively the present data show a positive result since 98.1 per cent. of the total offspring are of the grades expected.

Owing to the number of factors concerned, large numbers of family offspring, totalling 10,000 or more, are necessary in order to make an adequate quantitative test of the formula.

This can only be accomplished by the systematic and co-operative research of many workers, in different areas and populations.

Summary.

Two diverse sets of material have been used in an investigation of the inheritance of natural intelligence in man.

(1) The L.F. data of 194 Leicestershire families consisting of 388 parents and their 812 offspring, individually studied by the author.

(2) The R.F. data of the Royal families of Europe consisting of 212 families, including 424 parents and their 558 offspring, statistically studied by Woods (1906).

The concept of general intelligence is defined.

Following Galton (1869) and Woods (1906) individual parents and offspring have been graded for intelligence, on their general mental achievements, in 10 equal grades, from the highest grade 10 to the lowest grade 1. The grades are characterised as follows:—10, illustrious; 9, eminent; 8, brilliant; 7, talented; 6, able; 5, mediocre; 4, dull; 3, subnormal; 2, moron; 1, imbecile; (0, idiot). Equivalent juvenile gradings in terms of I.Q. are esti-

mated at 20 I.Q. per grade, *e.g.*, 200 I.Q. = grade 10, 100 I.Q. = grade 5, 20 I.Q. = grade 1.

The most frequent (grade 5) parents were found to be of three genetical types NN, Nn and nn, in which N is a dominant gene for normal mediocre intelligence (grade 5) and nn is a recessive pair of genes for abnormal variable intelligence in the presence of which any of the 10 grades of intelligence may be expressed. The 10 grades of intelligence are provisionally referred to the action of five pairs of minor genes Aa . . . Ee in the presence of the major pair nn. The dominant minor genes A . . . E act as equal and cumulative increasers of intelligence while the recessive minor genes a . . . e act as decreasers. In the presence of the dominant major gene N (NN or Nn) the minor genes Aa . . . Ee are inactive.

On this basis the genetical formula for the inheritance of intelligence is hexagenic, consisting of 1 major and 5 minor pairs of genes. It may be expressed in 729 genotypical forms, of which the most heterozygous form is [Nn + (Aa + Bb + Cc + Dd + Ee)].

It is concluded that the genetical formula proposed fits the diverse data sufficiently well qualitatively to be used as a working hypothesis for other families and populations, on which it should be tested quantitatively on a large scale by systematic and co-operative research.

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*A Manometric Analysis of the Metabolism in Avian Ontogenesis.**I.—The Normal Respiratory Quotient of Blastoderm, Embryo, and Yolk-Sac during the First Week of Development.*

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Introduction.

In a previous paper (Needham, 1932) the results of manometric experiments on the extra-embryonic membranes of the hen's egg were described. These experiments, however, were confined to the measurement of oxygen-uptake, their main interest centering in the degree to which the membranes participated in the metabolism of the whole egg, and did not include any measurements of the respiratory quotient. Difficult though the respiratory quotient may be to interpret, it must still be recognised as an essential preliminary to the analysis of the metabolism of a living system, and a considerable number of estimations of the quotient on the blastoderm, embryo, and yolk-sac of the chick during its first week of development have accordingly been made.

Investigations of the respiratory quotient of the *intact* egg have been made by several workers (Bohr and Hasselbalch, 1900; Hasselbalch, 1900; Lussana, 1906; and Murray, 1927; all summarised and discussed by Needham, 1931). They suffer from the disadvantage that until the last week of development, the amount of respiring protoplasm is small in relation to the mass of inert yolk, white, and shell, and the measurements are therefore exceedingly difficult. Nor can the non-living portions of the egg be truly called inert in this connection, for the egg-white certainly possesses an alkali reserve, and although attempts have been made to correct for this (*e.g.*, Needham, 1926), they do not lead to any satisfactory certainty.

In fig. 1 is plotted all the information at our disposal concerning the respiratory quotient of the intact egg. Points lying towards the end of the incubation-period may be regarded as accurate, since the greater part of the system is then living, but at earlier stages, this can certainly not be the case. This has been

shown on the graph by a difference in the diameter of the circles, which have been drawn on the assumption that a direct relation exists between the proportion of living matter present and the accuracy of the quotient. The large circles then, contrary to the common practice, represent the most accurate, not the most inaccurate, data. From fig. 1 it is clear that the first two-thirds of development remain an uncharted region, in spite of direct determinations of the respiratory quotient on the intact egg. It is also fairly clear that during the last week of development the respiratory quotient of embryo, yolk-sac, and

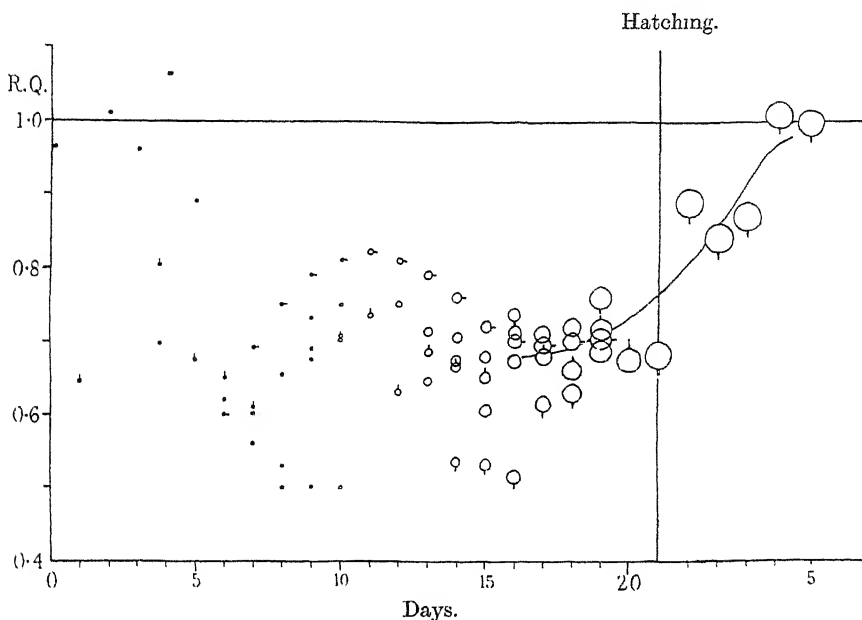


FIG. 1.—Respiratory quotient (intact egg).

○ Lussana ○ Bohr and Hasselbalch ○ Hasselbalch ○ Murray

The more accurate the determination the larger the circle. The sizes of the circles have been obtained by putting $d = w_1/w_2$ where d = diameter of circle, w_1 = weight of living matter in the system, w_2 = weight of inert matter including the shell.

allantois together, is in the close neighbourhood of 0.7, and that after hatching, or during the process, the quotient rises rapidly towards unity. As regards the first week of development, little can be said, save that then, and then alone, quotients higher than 0.8 were sometimes obtained by the earlier workers. In a subsequent paper the conditions during the last week of development will be discussed in detail; here only the experiments which have thrown light on the initial stages will be considered.

Experimental Methods.

The classical method for the determination of respiratory quotient *in vitro* was to compare the readings of two Barcroft differential manometers containing two similar pieces of tissue, but lacking in one case the potash. In this way the carbon dioxide production, and hence the respiratory quotient, could be calculated (as by Büchner and Gräfe, 1924). In 1926, Warburg, wishing to use a physiological concentration of bicarbonate in the suspending medium, introduced his "improved method" in which the respiration is calculated from the readings of two manometers containing similar pieces of tissue but different volumes of fluid. The result, which depends on the different solubilities of oxygen and carbon dioxide, is not of much value for calculating the respiratory quotient, because the carbon dioxide estimated includes that produced from the medium by the formation of lactic acid as well as that produced by true respiration in the tissue. Modifications of this method include analysis of the gas-phase in equilibrium with the solutions (Slater, 1926) and the absorption of the carbon dioxide by baryta in a vessel arranged for the measurement of electrical conductivity (Fenn, 1928).

All these procedures suffer from one fundamental disadvantage, namely, that they do not allow for the carbon dioxide chemically bound in the tissue or in the solutions. This may in certain cases be very small or negligible, but in others it is certainly not so, and in a later paper of this series, instances will be given in which the measurement of carbon dioxide production would be absolutely meaningless were no distinction made between bound carbon dioxide and true respiratory carbon dioxide. Crabtree (1929) indeed, has combined the bicarbonate method of Warburg already mentioned, with estimation of bound carbon dioxide by acidification before and after the experiment, but the fact that bicarbonate is artificially added to the medium makes the change of bound carbon dioxide required for calculating the respiratory quotient appear as a small difference between two large readings, and thus greatly limits its accuracy.

Measurement of respiratory quotient *in vitro* must involve, then, measurement of bound carbon dioxide, and up to 1930 this was done (*e.g.*, by Richardson, 1929) according to the old principle of Büchner and Gräfe, by using two manometers and two pieces of tissue. But even so, a fundamental source of error remained, namely, the assumption that the oxygen consumption and carbon dioxide production were identical in the two pieces of tissue used in the experiment, allowing for their weight. Whereas in certain cases, such an

assumption might be justifiable, it was certainly not so where tissues with low metabolic rate and variable protoplasmic content (such as the extra-embryonic membranes of the chick) were being used. Methods for working with only one piece of tissue were accordingly elaborated, independently and almost simultaneously by Meyerhof and Schmitt (1929) and by Dickens and Simer (1930). The former method is primarily designed for muscle and nerve, so the latter was adopted in the present work.

It depends essentially upon the presence of an annular space around the central chamber of the manometer cup in which the respiring tissue rests. The oxygen utilised by the tissue causes a diminution in pressure which can be followed on the Warburg manometer to which the cup is attached, and during this process, the carbon dioxide produced is being absorbed by a baryta solution in the annular space. At the end of the experiment, the acid in a side-bulb is tipped, and the carbon dioxide is driven off from both annular and central spaces, making itself apparent as a large positive pressure on the manometer. In an exactly similar manometer, the acid is tipped at the beginning

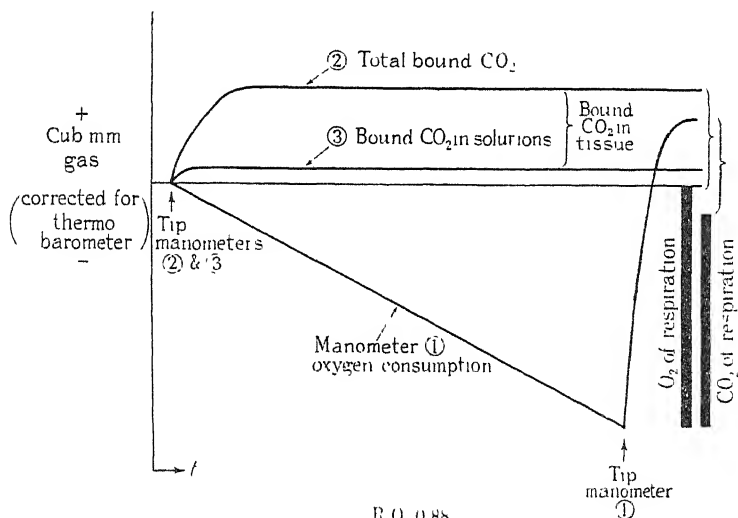


FIG. 2.—Diagram to show the principle of the method used to determine the carbon dioxide output of tissue.

of the experiment, thus giving the bound carbon dioxide in tissue and in solutions; and a third manometer, containing the solutions alone, will give a figure for the carbon dioxide content of the solutions alone. The operation of a typical experiment is shown in fig. 2. A steadily increasing negative pressure in manometer 1 indicates the oxygen-consumption of the tissue. The positive

pressure in manometers 2 and 3, which have been acidified at the beginning of the experiment, indicate the bound carbon dioxide in tissue plus solutions, and in solutions, respectively, hence that in the tissue can be found by subtraction. At the conclusion of the experiment, the manometer 1 is acidified, with the result that a positive pressure is produced due to respiratory carbon dioxide, carbon dioxide in tissue, and carbon dioxide in solutions. Since the latter two components are known, the former can be calculated, and when compared with the oxygen-consumption, gives, in the hypothetical example chosen for the diagram, a respiratory quotient of 0.88.

It may be convenient to describe at this point the procedure in a typical experiment. 10 c.c. of Ringer solution (NaCl 1.04, CaCl_2 0.02, KCl 0.02 gm. per 100 c.c. giving $\Delta - 0.6^\circ$) plus 0.2 c.c. of a 10 per cent. glucose solution are put to boil in a small conical flask for 15 minutes to remove as much carbon dioxide as possible, and another quantity of 25 c.c. of the Ringer solution is boiled at the same time separately. Meanwhile the stoppers and necks of the manometers are carefully greased with pure adeps lanae. The Ringer and Ringer-glucose solutions are cooled in a stream of oxygen. During the cooling, the eggs are opened, and the embryos dropped into a Petri dish of Ringer solution at 37° in which their amnion and allantois are removed before they are weighed on tarred watch-glasses. Or if yolk-sacs are required, they are freed from adherent yolk in the manner previously described, and weighed. Stock CO_2 -free sodium phosphate solution (2.43 dry NaH_2PO_4 and 12.68 Na_2HPO_4 gm. per litre, *i.e.*, 0.109 M) is now added to the Ringer-glucose to the amount of 3 c.c. making a total of 13.2 c.c. at p_{H} 7.4. The Ringer-glucose solution must be cold before the phosphate is added. Next the Ringer solution is divided into as many beakers as there are pieces of tissue, and the stream of pure oxygen continued in the presence of the tissue for about a quarter of an hour in order to equalise the bound carbon dioxide as far as possible. At the end of this time, the oxygenated Ringer-glucose-phosphate is pipetted out into the central chambers of the manometer cups (2 c.c. per cup)* and 0.3 c.c. of 2.5 N HCl added to each side-bulb. The stream of oxygen is intensified and diverted so that it passes through the manometers and fills the cups immediately they are attached with their tissue and solutions. The manometers are now in turn brought underneath a burette containing saturated baryta (0.3 N), so that 0.5 c.c. of the baryta can pass into the annular space

* The pipette used for this is provided with a guard-bulb filled with soda-lime, and itself forms the tube through which oxygen is bubbled through the Ringer-phosphate-glucose.

without ever coming in contact with the atmosphere of the room. The stoppers of the cup are now pushed home, the manometer tap is turned, and the whole apparatus is ready to be placed in the thermostat bath. When not in use the mouth of the baryta burette should be protected by immersion in N/20 HCl.

The bath used in the present experiments was considerably larger than the usual Warburg bath, and was maintained at any desired temperature by an ether-mercury thermostat. Shaking was accomplished in the usual way for Warburg manometers. Equilibration to the bath temperature was considered to be complete after 10 minutes. It is important to see that the manometer taps are open before the cups are placed in the bath, and to work in the stoppers of the cups during the 10 minutes of equilibration. If this is not done the attainment of an appreciable negative pressure will drag water in from the bath however well the stoppers have been greased.

The actual amount by volume of gas given off or consumed (cubic millimetres) is calculated by multiplication of the pressure change observed (millimetres Brodie fluid in the manometer) by a constant whose value depends on the vessel-volume and the nature of the gas. The vessel-constant, k , was calculated from the formula

$$k = \frac{V_g \frac{273}{T} + V_{f.a}}{9986},$$

where V_g is the volume of gas in the cup (measured by filling with mercury and weighing afterwards), V_f the volume of fluid (2800 cub. mm. in the present experiments), T the absolute temperature of the bath employed, α the Bunsen solubility coefficient of the gas in question, and the denominator the specific gravity of Brodie fluid (see Warburg, 1926; and Dixon, 1932). Each manometer cup therefore has two constants, one for oxygen and one for carbon dioxide.

In order to demonstrate the working of the method on the material used, two protocols may be given in full. Table I shows respiratory quotients of 5th day yolk-sac in Ringer-phosphate-glucose, and Table II is of special interest as it refers to experiments made on embryos in amniotic liquid plus glucose. The bound carbon dioxide is here relatively much larger in proportion to the whole than in Table I, but it was thought undesirable to boil the amniotic liquid since the greatest possible similarity to *in vivo* conditions was the aim.

Table I.—Protocol. Yolk-sac; washed in boiled oxygenated Ringer; temperature 39.5°.

Experiment number	216	217	218	219	220
Apparatus	1	2	4	6	7
Total volume in c.c.	24.838	24.161	24.801	24.057	23.732
C.c. 2.5 N HCl in side-bulb	0.3	0.3	0.3	0.3	0.3
C.c. Ringer-phosphate-glucose in central part	1.75	1.75	1.82	1.8	2.0
Gm. tissue { 5th day	0.251	0.245	—	—	—
{ 5½th day	—	—	0.187	0.213	—
C.c. baryta in annular trough	0.5	0.5	0.5	0.5	0.5
Acid tipped after 10 minutes in bath	No	Tip	Tip	No	Tip
Pressure changes corrected for thermobarometer between following times (zero hour, 1.5 p.m.) in c.c. Brodie fluid—					
1.8	— 0.25	1.05	1.25	0.25	0.75
1.14	— 0.2	0.1	0.1	— 0.25	0.1
1.30	— 0.95	—	—	— 0.9	—
1.50	— 1.15	—	—	— 1.15	—
2.15	— 1.45	—	—	— 1.45	—
2.47	— 1.45	—	—	— 1.7	—
3.30	— 1.8	—	—	— 1.95	—
3.45	— 0.55	—	—	— 0.7	—
4.5	— 0.7	—	—	— 0.8	—
5.15	— 2.35	—	—	— 3.0	—
5.50	— 0.95	—	—	— 1.2	—
6.20	— 0.65	—	—	— 0.85	—
6.23	Tip	—	—	Tip	—
6.39	+ 10.35	—	—	+ 11.35	—
6.45	—	—	—	—	—
Calculation—					
h_{O_2}	—12.4	—	—	—14.2	—
k_{O_2}	19.6	—	—	18.8	—
Total oxygen consumed (cub. mm.)	242	—	—	267	—
h_{CO_2}	10.35	1.15	+ 1.35	+ 11.35	+ 0.85
k_{CO_2}	21.2	20.4	21.0	20.3	20.0
Total carbon dioxide given off	220	23.5	28.0	231	17
Correction for tissue and solutions	196.5	—	—	199	—
R.Q.	0.82	—	—	0.75	—

N.B.— h_{O_2} and h_{CO_2} = the differences between the readings on the manometer, in cm. Brodie fluid. k_{O_2} and k_{CO_2} = the vessel constants.

In the course of many experiments with the method an unexpected phenomenon was encountered. Theoretically, all pressure-changes should cease after acidification, as is seen in Tables I and II, but in certain experiments this did not occur, and a very slowly increasing negative pressure manifested itself after the acidification of the tissue. This effect must in all probability be due to oxygen consumption without carbon dioxide production, since after tipping, no more absorbent for CO_2 remains in the cup, and if anything like normal respiration had been going on, the gas exchange would have largely cancelled itself out. A similar phenomenon has been described by Amberson,

Armstrong and Root (1931) who found that *Fundulus* eggs and embryos after acidification showed an oxygen consumption amounting to 14 per cent. of the normal, but no carbon dioxide production. These workers did not offer any explanation of their results, but it is sure that living or moribund material must contain autoxidisable substances, and it is very conceivable that any or

Table II.—Protocol. 5th day embryo ; washed in boiled oxygenated Ringer ; suspension medium 9th day unboiled amniotic liquid with glucose to make 0.2 per cent. ; temperature 37°.

Experiment number	445	446	447
Apparatus	1	6	7
Total volume in c.c.	24.838	24.057	23.732
C.c. 2.5 N HCl in side-bulb	0.3	0.3	0.3
C.c. amniotic liquid in the central part	1.7	1.65	1.7
Number of embryos	3	4	3
Gm. wt. of embryo tissue	0.308	0.359	0.309
C.c. baryta in annular trough	0.5	0.5	0.5
Acid tipped after 10 minutes in bath	Tip	No	No
Pressure changes corrected for thermobarometer between following times (zero hour 12.25 p.m.) in c.c. Brodie fluid—			
12.30	+ 4.6	— 1.3	— 1.25
12.57	—	— 5.05	— 4.65
1.50	—	— 9.0	— 7.9
2.5	—	— 2.2	— 1.85
2.6	—	Tip	Tip
2.10	—	+21.65	+18.95
2.35	—	—	—
Calculation—			
h_{O_2}	—	—17.55	—15.65
k_{O_2}	—	18.8	18.5
Total oxygen consumed (cub. mm.)	—	330	290
h_{CO_2}	+ 4.6	+21.65	+18.95
k_{CO_2}	21.2	20.3	20.0
Total carbon dioxide given off (cub. mm.)	97	441	379
(Correction for tissue and solutions	—	328	282
R.Q.	—	0.99	0.98

all of the following processes may be going on after acidification in the manometer cup with its atmosphere of pure oxygen (*a*) the oxidation of organic sulphur to sulphate, (*b*) the transformation of lactic acid to pyruvic acid or of glucose to glycuronic acid. (*c*) the formation of acetoacetic acid from fat, (*d*) the oxidation of reduced glutathione, (*e*) the oxidation of unsaturated lipoids.

It is legitimate to assume that this "autoxidation" oxygen is also involved in the manometers where normal respiration is proceeding, and it should therefore be subtracted from the oxygen due to true respiration just as the bound carbon dioxide is subtracted from the carbon dioxide due to true respiration. The fundamental formula is:—

$$\frac{x}{y - (a + b)},$$

where x is the oxygen-uptake, y the total carbon dioxide recovered, and a and b the bound carbon dioxide in tissues and solutions respectively. Allowing for the autoxidation oxygen, c , it becomes

$$\frac{x - c}{y - (a + b)},$$

But, since c , as described above, is determined on the same manometer as a and b , it is convenient to subtract it direct from them,

$$\frac{x}{y - (a + b - c)}.$$

This formula will not give precisely the same result as the correct one, but since a , b and c are small relatively to x and y , the error is not appreciable and does not affect the interpretations which can be drawn from the quotient.

The legitimacy of allowing for the autoxidation oxygen depends, of course, on the assumption that it has not been unduly favoured by the conditions of acidity prevailing in the manometer after tipping. Unfortunately it is not easy to obtain information about the effect of p_H on the various factors listed above, but in the case of the oxidation of reduced glutathione, at least, it was shown by Dixon and Tunnicliffe (1923) that the velocity falls off greatly on the acid side of neutrality. The autoxidation oxygen would therefore tend to be less in the acidified tissue than in the tissue respiring under normal conditions of p_H .

A typical instance may be given (experiment 357, a 5-day old chick embryo in Ringer-phosphate-glucose medium, atmosphere pure O_2 , temperature 36°). The bound CO_2 in the tissue and solutions gave a positive pressure at the beginning of the experiment but during the succeeding period of 4 hours 35 minutes, during which the main manometer was developing a negative pressure of -6.95 cm. Brodie fluid, the acidified cup also developed a negative pressure amounting to -0.55 cm. As the weights of the embryos were almost identical, the autoxidation oxygen consumption was about 8 per cent. of the total oxygen consumption, *i.e.*, a "residual" oxygen consumption of exactly the same order as that reported by Amberson, Armstrong and Root.

With regard to the preparation of the living material there is little to say, except in the case of the blastoderms of less than $2\frac{1}{2}$ days development. Incubation of the eggs was carried out according to the standard conditions laid down by Murray (1925) and Needham (1926). They were all derived from the

pure-bred White Leghorn flocks of Messrs. Chivers and of the Cambridge University Farm.

The blastoderm has not hitherto been the subject of any physiological or manometric experiments, for it is much too fragile to withstand prolonged shaking, and readily fragments in physiological salt solutions. This disadvantage was overcome, however, by attaching the blastoderm to a piece of silk by means of a clot of adult plasma, and then placing the whole system in the manometer cup. The procedure is as follows:—The egg is broken into an evaporating basin and the egg-white poured off. The yolk is then floated into a Petri dish filled with warmed Ringer and so manoeuvred that the germinal area lies uppermost just underneath the surface of the Ringer solution. The area is then isolated by cutting round it with a pair of sharp scissors and the blastoderm floated off upside down. If the operation is conducted over a black tile, it is easy to clean the blastoderm from adherent yolk with an iridectomy knife and then to float it right side up on to a silk disc. These discs are about 2.5 cm. in diameter and are cut out of commercial white Chryside silk. The disc is now removed from the Petri dish and laid upon a glass plate, a couple of drops of adult avian plasma added, and the whole left to clot, a process which is complete in from 10 to 15 minutes. The plasma is obtained by centrifuging carotid blood, and decanting the plasma into ice-cold paraffined tubes, leaving the buffy coat on the corpuscles. The silk disc should be saturated with plasma before being immersed in the Ringer solution. It is extremely important that the vitelline membrane should not be removed from the blastoderm, but should be left in place; if it is removed, the clot alone may not be sufficient to keep the blastoderm in place throughout the experiment, and the *area pellucida* may be washed away. It was found that very early blastoderms (before the appearance of the primitive streak) were not large enough to make the plasma clot properly, but the addition of a drop of 10th-day embryo extract quickly remedied this.

In order to have some guide as to the relation between age, number of somites, axial length, etc., the data contained in Lillie's "The Development of the Chick" (1927) were plotted in the form of a graph, and any embryos which did not conform to this scale were rejected as abnormal. Weighing of the blastoderms presented very serious difficulties which up to the present time have not been overcome, for any process which removes water from the material sufficiently to enable an accurate wet weight to be obtained also irreparably injures the tissue. Some figures were collected by weighing the blastoderm *in situ* on its silk disc on a torsion balance, but their accuracy was probably

not great, and as the respiratory quotient is established on one single blastoderm, further search for a satisfactory weighing method was laid aside for the present. The comparison between blastoderms necessitated by the bound carbon dioxide control was made on the basis of area.

As regards the physiological nature of the suspending medium employed, little doubt need be felt since Dickens and Simer (1931) have shown that the respiratory intensities and the respiratory quotients of a large variety of tissues (including the chick embryo) are exactly the same whether determined in phosphate or bicarbonate. It is true that Lyon (1927) and Barmore and Luck (1931) have shown that the phosphate ion is not without a specific affect on certain enzyme actions, but so much work has already been done on cell-respiration in Ringer solutions buffered with phosphate, that for comparative purposes, its use is inevitable at present.

It might, however, be questioned whether any harmful effect (such as the washing out of metabolites) was produced by the 15-minute washing to which all the tissues were subjected in order to equalise and reduce to a minimum the bound carbon dioxide. Table III shows that such washing does somewhat diminish the oxygen-consumption of the yolk-sac, but that the effect is entirely rectified by the addition of glucose to the suspending medium in the manometer cup.

Table III.

All figures in cubic millimetres per hour oxygen-uptake per gram for the first 2 hours.

Experiment number.	Day of development.	Without glucose.			With glucose.		
		Unwashed.	Washed.	Per cent. diminution.	Unwashed.	Washed.	Per cent. diminution.
3, 4	4	334	183	45	—	—	—
110, 111	4	—	—	—	333	447	None
135, 136	6	—	—	—	200	185	7.5
137, 138	6	290	180	38	—	—	—
147, 148	6	—	—	—	129	211	None
149, 150	6	220	173	22	—	—	—
154, 155	6	—	—	—	426	363	14.8
156, 157	6	357	297	17	—	—	—
93, 94	15	—	—	—	262	272	None

Experimental Results.

The results obtained with the foregoing methods are summarised in Tables IV, V and VI, and in fig. 3. Considerable difference is immediately apparent

Table V.—Normal Respiratory Quotient of Embryo.

Experi- ment No.	Age in days.	Morphological development.	Tempera- ture.	Medium.	Time of experi- ment.	No. of embryos taken.	Wet weight mgms.		Cub. mm. oxygen consumed.	Cub. mm. carbon dioxide given off.	Cub. mm. bound carbon dioxide.	R.Q.
							Total.	Each.				
319	2.5	30 somites	T° 37.0	RPG	h. m. 3 57	2	6	3	13.0	29.5	17.0	0.96
337	2.5	30 "	37.0	RPG	4 36	5	30	6	76.5	87.0	7.0	1.04
338	2.5	30 "	37.0	RPG	4 36	5	28	6	79.0	91.0	7.0	1.06
158	3.0	—	38.7	RPG	6 9	4	93	23	232.0	261.0	31.0	0.99
446	5.0	—	37.0	AML	1 40	4	359	89	330	441	113	0.99
447	5.0	—	37.0	AML	1 40	3	309	103	290	379	97	0.98
309	6.0	—	37.0	RPG	3 15	1	129	129	202	223	17	1.00
310	6.0	—	37.0	RPG	3 15	1	112	112	242	260	20	1.00

RPG = Ringer-phosphate-glucose.

AML = Amniotic liquid.

Table VI.—Normal Respiratory Quotient of Yolk-sac ; medium Ringer-phosphate-glucose.

Experiment No.	Age in days.	Temperature.	No. taken.	Wet weight mgms.		Time of experiment.	Cub. mm. oxygen consumed.	Cub. mm. carbon dioxide given off.	Cub. mm. bound carbon dioxide.	R.Q.
				Total.	Each.					
151	3	38.4	1	182	182	h. m. 5 46	334	316	24	0.88
144	4	38.4	1	355	355	2 39	305	296	43	0.84
216	5	39.5	1	251	502	5 18	242	220	17	0.84
219	5	39.5	1	213	426	5 18	267	231	32	0.75
165	6	38.4	1	288	576	4 36	436	384	32	0.81
176	6	38.4	1	477	954	1 53	367	307	32	0.75
221	6	39.5	1	297	594	3 20	293	228	22	0.70
223	6	39.5	1	387	774	3 20	359	296	22	0.76
227	8	39.5	1	281	843	3 0	318	229	40	0.60
228	8	39.5	1	292	876	3 0	206	145	31	0.55

between the data for embryo and for yolk-sac, for whereas the respiratory quotient of the former (up to the 6th day, after which time the intact embryo becomes too large for such measurements) is in the close neighbourhood of unity, that of the former is never higher than 0.9. The results for the embryo are fully confirmatory of the earlier work of Dickens and Simer (1930). Moreover, the respiratory quotient of the yolk-sac is not constant with time, but falls from 0.9 on the 2nd day of development to 0.6 or below on the 8th. Thus the metabolism of the embryo must be remarkably different from that of its membranes, and it would seem that the combustion of proteins and fats begins much sooner in the latter than it does in the former. The solid line on the extreme right of fig. 3 shows the respiratory quotient of the entire egg at the end of development, and is copied from fig. 1. We must assume that soon after the 6th day a lowering of the embryo's respiratory quotient takes place, bringing its tissues into line with the metabolism of the yolk-sac, and the probable course of the respiratory quotient during the middle portions of development is therefore represented by dotted lines in fig. 3. Evidence concerning the respiratory quotient of the yolk-sac during the last 2 weeks of development will be presented in a subsequent paper of this series, but parallel information for the embryo will not be forthcoming so long as we confine ourselves to the study of the intact cells and avoid the traumatism associated with section-cutting, mincing, and so on.

The respiratory quotients of the early blastoderms were distinctly variable, but this variability had an obvious explanation in the light of the difference between the quotients of later embryo and later yolk-sac. The blastoderm is

composed of *area pellucida* (with a metabolism which we may assume to be the same as that of the later embryo) and of *area opaca* (with a metabolism which we may assume to be the same as that of the later yolk-sac). If this view is adopted, it is not surprising to find that variability should occur, for the quotient of a blastoderm would depend on the relative amounts of *area pellucida* and of *area opaca* present. In support of this conception are the results of certain experiments, shown in Table IV, in which the *area pellucida* was removed before the blastoderm was stuck to its disc. In all these cases, the quotient turned out to be lower than usual, and is represented on fig. 3 by a cross. A chance abnormality which was encountered, namely, an Anidian blastoderm, gave further support to this view. An Anidian blastoderm (see Dareste, 1877 ; Tur, 1929 ; and Grodzinski, 1931) is one in which, although a good deal of proliferation has gone on, there is no *area pellucida* and no embryo. On this a very low quotient was obtained.

The results here reported, then, have filled in the unexplored region before the 10th day (see fig. 1). They are in general agreement with the conception of the succession of energy-sources previously described (Needham, 1931), but they show that the rhythm of this succession takes place differently in embryo and yolk-sac, the combustion of protein or fat occurring earlier in the latter than in the former. The evidence strongly suggests that this characteristic is developed at a very early stage of development, the cells of the *area pellucida* following an "embryo" course, and those of the *area opaca* following a "yolk-sac" course. Experimental modification of these processes will form the subject of the second paper of this series.

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Summary.

(1) Manometric methods have been adapted for the determination of the respiratory quotient of the chick embryo and yolk-sac *in vitro*. By the use of plasma clot and a supporting disc of silk, the respiratory quotient of blastoderms at all stages of development may be obtained.

(2) The respiratory quotient of the embryo from 2½ to 6 days of development is nearly unity, whether measured in phosphate or bicarbonate Ringer solution or in amniotic liquid.

(3) The respiratory quotient of the yolk-sac falls from 0.9 at $2\frac{1}{2}$ days of development to a little below 0.6 at 8 days.

(4) Since low quotients are obtained from blastoderms in which the *area pellucida* is either experimentally or naturally absent, it is concluded that the *area pellucida* resembles the later embryo in giving a high quotient (0.95 to 1.0), and the *area opaca* resembles the later yolk-sac in giving a low one (below 0.9). Up to $2\frac{1}{2}$ days of development, the blastoderm has a respiratory quotient varying between 0.85 and a little above unity.

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A Manometric Analysis of the Metabolism in Avian Ontogenesis. -
 II. *The Effects of Fluoride, Iodoacetate, and other Reagents on the*
Respiration of Blastoderm, Embryo, and Yolk-Sac.

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Introduction.

The study of cell-respiration by means of agents with a known action upon some one part of the cellular mechanisms has in recent years been much pursued, and it has to some extent been possible to make a differential attack upon the various co-operating processes which are so efficiently integrated in the intact cell. These methods suffer, it is true, from the disadvantage that the addition of an ion such as fluoride to the cell-interior may not merely inhibit a certain reaction which normally goes on there, but may also bring into being a number of distinctively pathological reactions which have no place in the normal cell. It is not surprising, therefore, that the results of experiments on cell-respiration are difficult to interpret. But difficulties of interpretation are no ground for failing to make use of any methods which are available, and the possibility of complicated secondary effects is, after all, common to all biological methods in which the normal course of events within the organism is interfered with.

Up to the present time, the study of the effect of agents such as fluoride, cyanide, iodoacetic acid, triphenylmethane dyes, sulphides, pyrophosphates, etc., has been confined to the cells of adult tissues (*cf.* Dixon, 1929; Wurmser, 1930) or to bacteria (*e.g.*, Haldane, Cook and Mapson, 1931). But it would obviously be of much interest to observe their effects upon cells of early embryonic stages for we might hope in this way to discover something of the way in which the chemical machinery of the cell is laid down. To what extent, for instance, do the cells of a somite in a two-day old chick embryo resemble adult muscle cells in their reactions to the glycolysis-inhibiting action of iodoacetic acid? Moreover, a good deal of evidence exists that embryos in the earlier stages of development combust carbohydrate molecules exclusively, and that later on the combustion of protein and fat sets in. What will happen,

then, to the metabolism of embryonic cells in the carbohydrate stage if their glycolysing power is artificially inhibited? Is the power of deaminating and combusting amino-acids already present and not used, or has it not yet developed? In the former case the respiratory quotient should betray a change over to protein combustion; in the latter case the cells should cease to respire altogether. It was to answer questions such as these that the work described in the present paper was undertaken.

Experimental Methods.

The manometric methods employed have already been described in the first paper of this series. For respiratory quotient determinations Warburg manometers fitted with Dickens-Simer phosphate cups were used, and where the oxygen-uptake alone was desired, Barcroft differential manometers. For the details of the technique, see Dickens and Simer (1930) and Dixon and Elliott (1930). For tipping in a reagent during the course of an experiment, small glass cups hung on to the potash carrier in the Barcroft manometer were used. A small bent platinum wire permits of their being shaken off into the suspension medium whenever desired (Dixon, 1932).

All the reagents, such as fluoride and iodoacetate, were made up immediately before use in distilled water carefully freed from carbon dioxide. Sodium lactate was prepared by diluting a known amount of lactic acid and titrating it slowly while boiling against normal soda, using phenol-phthalein as the indicator. The iodoacetic acid was added to the Ringer-phosphate-glucose medium in such concentration as to have no effect upon the p_H of the latter, which was in all cases 7.4.

Ammonia estimations were carried out by the distillation method of Stanford (1923) as modified by Holmes and Watchorn (1927).

The Effect of Iodoacetic Acid.

(a) *On the Embryo.*—It was soon found that the respiration of the fourth or fifth-day chick embryo was completely inhibited after a moderate dose of iodoacetic acid. A typical experiment is shown in fig. 1, where a state of almost complete inhibition has set in about 3 hours after the beginning of treatment with the drug. The dose here used (0.3×10^{-3} M) was selected on the basis of the experiments of Meyerhof and Boyland (1931), who found, using adult amphibian muscle, that 0.25×10^{-3} M gave a 90 per cent. inhibition of lactic acid production, but only a 20 per cent. inhibition of respiration in Ringer-

lactate solution. Respiration might therefore have been comparatively unaffected if it were possible for the embryonic cells to burn something other than sugar, assuming always the correct dosage to be comparable. But in fact it was reduced after a certain time to nothing. The latent period effect, which is well shown in fig. 1, can hardly be due wholly to difficulty of penetration, since it was not altered by using third or fifth-day embryos, although the differences in size were very considerable (experiments 172 to 175). The

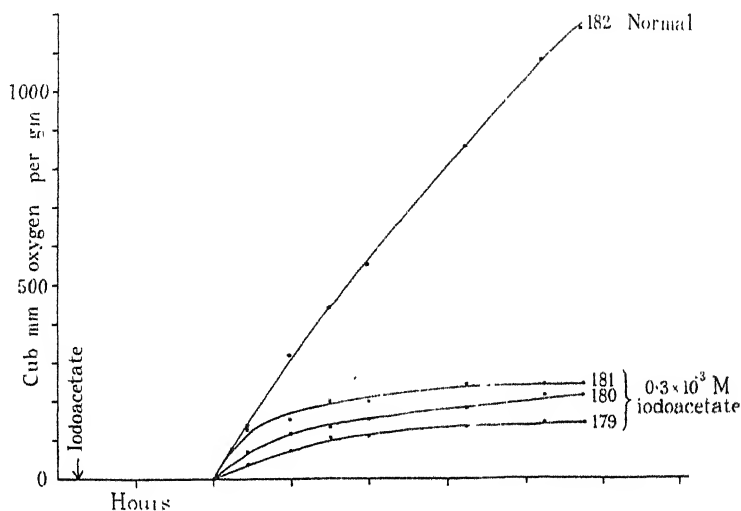


FIG. 1.—Experiments 179 to 182; 4th-day embryos.

latent period in the action of iodoacetic acid has often been observed by other workers. Thus Lohmann (1931) found that with $N/5000$ iodoacetic acid complete inhibition of lactic acid production in adult muscle was only obtained after two hours incubation with the drug.

If the combustion of fat were a possibility for the fourth-day chick embryo, there seems no reason why the respiration should cease, as in fig. 1. The dry weight of a fourth-day embryo is about 3 mgm. and according to the data of Murray (1926) and Cahn (1928) it should contain about 0.3 mgm. of fat. This would account for 605 cub. mm. of oxygen if the whole of it were burned, whereas each embryo in these experiments only took up some 70 cub. mm. of oxygen before coming to a stop, and of this amount a certain proportion, at least, must have been due to glucose katabolism until the iodoacetic acid had taken effect.

Further experiments (experiments 321 to 328) showed that the complete inhibition could not be entirely due to an effect of iodoacetic acid on the

oxidising mechanisms, for if lactate was added to the embryos after a certain amount of inhibition had occurred the respiration was restored. This is shown in fig. 2. The amount of lactate added was 5 mgm. and was contained in 0.2 c.c. of fluid in the cup hung to the potash carrier. Similar restoration was reported by Meyerhof and Boyland (1931) on adult frog muscle, and by Krebs (1931) on adult rat brain and testis. It is significant, as we shall see later, that these tissues possess a normal respiratory quotient of nearly unity (Dickens and Simer, 1930, *b*). According to Barrenscheen, Braun and Deguss (1931) methylglyoxal is as effective as lactic acid in this restoration.

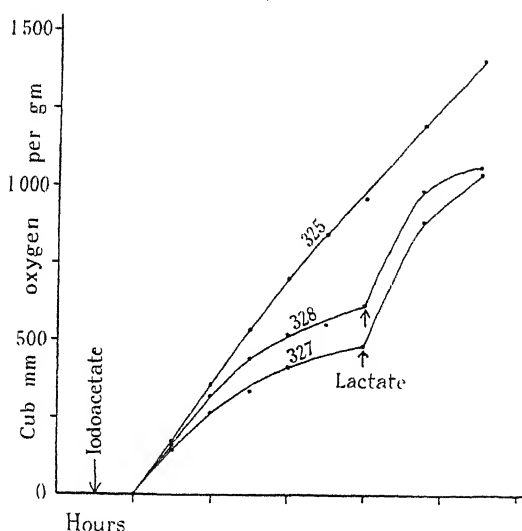


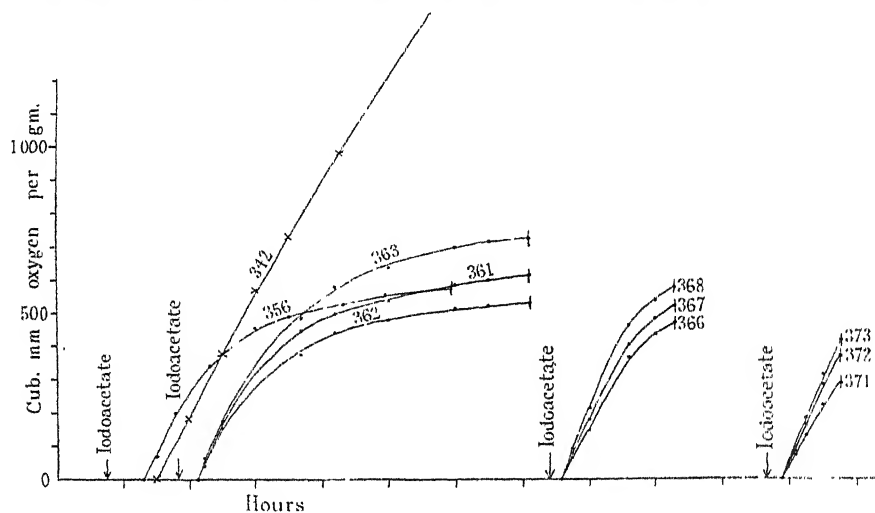
FIG. 2.—Experiments 325 to 328. Embryo lactate after 0.3×10^{-2} M. iodoacetate.

Experiment.	Days of development.	No. of embryos.	Mgm. wet weight.	
			Total.	Each.
328	4.5	1	50	50
325	5.0	2	154	77
327	5.5	1	129	129

The respiratory quotient of the embryos after iodoacetate treatment was next investigated. As is shown by Table I and fig. 3, it was found to be uniformly 0.84, in great contrast to the normal respiratory quotient of unity reported in the first paper of this series. It was at first thought that this quotient might be the resultant of two periods, a preliminary period before

Table I.—Respiratory Quotients of Embryo after Iodoacetate; Ringer-phosphate-glucose Medium.

Experiment No.	Age in days.	Temperature, ° C.	No. of embryos taken.	Wet weight mgm.		Time from turning taps.	Cub. mm. oxygen consumed.	Cub. mm. carbon dioxide given off.	Cub. mm. bound carbon dioxide.	R.Q.
				Total.	Each.					
						h. m.				
356	5	36	2	239	119	4 37	136	145	32	0.84
361	5	36	3	230	76	4 56	137	140	26	0.84
362	5	36	3	230	73	4 56	121	126	25	0.84
363	5	36	2	234	117	4 56	167	164	26	0.82
366	5½	36.5	1	267	267	1 42	125	147	44	0.82
367	5½	36.5	2	373	187	1 42	194	222	58	0.85
368	5	36.5	3	348	116	1 42	200	207	55	0.76
371	5½	36.5	1	230	230	0 51	65	66	44	0.34
372	5	36.5	2	239	119	0 51	87	104	46	0.67
373	5	36.5	2	251	125	0 51	99	107	47	0.60

FIG. 3.—Respiratory quotients of 5 and 5½-day embryos after 0.3×10^{-3} M. iodoacetic acid.

inhibition had set in, in which the normal quotient of unity was maintained, and a final period, after inhibition had set in, in which the quotient, owing to incomplete combustions, or some other reason, was much reduced, *e.g.*, to 0.3. Direct determination of the respiratory quotient on the late, inhibited, period was very difficult owing to the then small turnover of gases relative to the weight of the tissue, so attempts were made to obtain quotients for the early period. Contrary to expectation, no signs of a normal high quotient

could be obtained when the time of the experiment was shortened ; as fig. 3 shows, in experiments 366, 367 and 368, where the time elapsing after the turning of the taps was only 1 hour 42 minutes, the respiratory quotients were still 0.76, 0.82 and 0.85. When the time was still further shortened, as in experiments 371 to 373, the quotients came even lower, but here (a duration of only 51 minutes) the gas exchange due to true respiration was too small in relation to the bound carbon dioxide to permit of much accuracy in the calculation. While no emphasis can be laid on the exact values of the quotients 0.34, 0.60 and 0.67, they do at any rate show that even in the first hour after the tissue has come in contact with iodoacetate, the respiratory quotient is considerably lower than unity.

It is questionable whether this indicates the combustion of molecules other than sugar. The path of breakdown might be clear up to the stage of formic acid, for example, but not after that point (see D. M. Needham (1932), p. 105). If this were so, iodoacetic acid would be exerting two effects on the embryonic cells, one upon some point in the oxidative mechanisms and subject to no latent period, and one upon glycolysis, subject to a latent period. Respiration is only checked when the second of these actions is coming into play.

These results make an interesting comparison with those of Meyerhof and Boyland (1931). After iodoacetic acid poisoning the normal respiratory quotient of amphibian muscle (0.95) is depressed to between 0.7 and 0.8, although 70 per cent. of the normal oxygen-uptake still continues. Given lactate, the respiration rises and the respiratory quotient returns to normal. These results have generally been interpreted as meaning that a combustion of protein or fat takes place during the inhibition period. As regards the embryo, however, Glover (1931) states that chick embryos of 4 to 5 days incubation exercise no deaminating power upon amino-acids *in vitro*, when tested directly by estimation of ammonia and urea. The question will be taken up again in the discussion after the work on fluoride effects has been described.

Very recently, Landsgaard (1932) has shown that yeast in which the glycolysis has been totally suppressed by iodoacetate will continue to respire for a long period with a respiratory quotient of unity. This result, which closely resembles the phenomena seen when the chick embryo is poisoned with fluoride (see below) he attributes partly to the oxidation of products formed from alcohol, and partly to the existence of a path of breakdown for glucose not involving lactic acid as an intermediary. He does not envisage the possibility of ammonia formation.

(b) *On the yolk-sac.*—The effect of iodoacetic acid on the respiration of fourth-day yolk-sac (experiments 161 to 164, 168 to 171 and 197 to 200) was very similar to that on the embryo, but the final inhibition did not seem to be quite so complete. In some cases a steady uptake of about 24 cub. mm. oxygen per gram wet weight per hour was still going on at the conclusion of the experiment (6 or 7 hours after the first contact with iodoacetic acid). But the most striking thing about the inhibition of the yolk-sac respiration was that it was not removed by the addition of lactate (see fig. 4, experiments 197 to 200). This finding is in agreement with the generalisation made by Dickens (1932) that tissues having a normal respiratory quotient of unity can oxidise

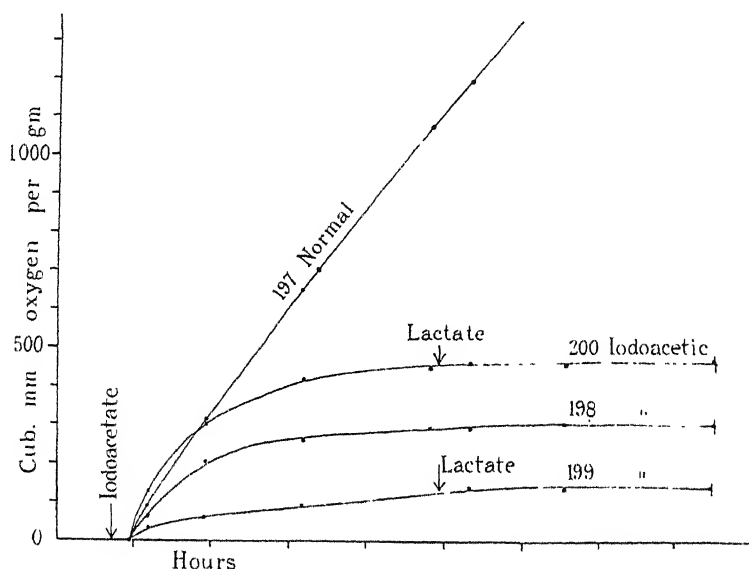


FIG. 4.—Effect of iodoacetic acid on respiration of yolk-sac.

added lactate after iodoacetic inhibition, while those with a normal respiratory quotient of 0.8 or lower cannot do so. It is clear that although of the same age embryologically, the embryo and the yolk-sac have embarked upon a very different line of metabolism.

(c) *On the Blastoderm.*—The effect of iodoacetic acid (0.3 and 0.4×10^{-3} M) on the respiration of the blastoderm was uniformly inhibitory, and the latent period required for complete cessation of oxygen-uptake was much less than in the case of embryo or yolk-sac. Regeneration of the respiration by added lactate, however, was variable, sometimes taking place and sometimes not (experiments 266 to 269 and 270 to 273). Fig. 5 shows four experiments, in two of which no restoration of oxygen-uptake occurred when lactate was

added, but in one of which a positive result was obtained. It seems probable that this variability may be referred to the same cause as the variability in normal respiratory quotient shown by blastoderms and commented on in the first paper of this series. The blastoderm in the manometer cup is not a homogeneous entity but consists of *area pellucida* and of *area opaca*, and if we may assume, as seems very justifiable, that the former approximates to embryo from a metabolic point of view, and the latter to yolk-sac, then the blastoderm will show the properties of either depending on which of its constituent parts is the more active or the less injured by the experimental conditions.

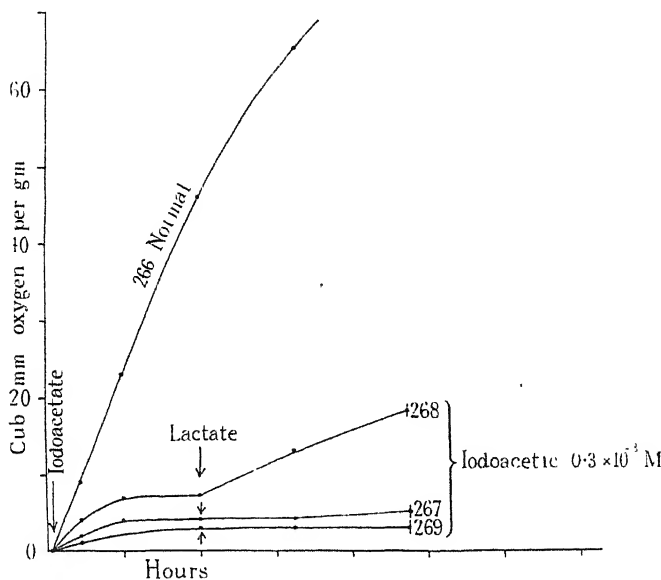


FIG. 5.—Effect of iodoacetic acid on the respiration of the blastoderm ($1\frac{1}{2}$ days, 15 somites); experiments 266 to 269.

The Effect of Fluoride.

(a) *On the Embryo*.—The first phenomenon which was encountered when the effect of fluoride on embryonic respiration was studied was that concentrations theoretically sufficient to stop glycolysis altogether had no effect on the respiration. It has been known for some time that the dosage for inhibition of glycolysis differs considerably from that needed for inhibition of respiration, and the evidence on which this statement is based has been summarised in Table II, but as will be seen from fig. 6, concentrations of sodium fluoride which should have given 100 per cent. inhibition of glycolysis had no appreciable influence on the respiratory rate (experiments 329 to 332 and 333 to 336).

Table II.—Dosages of Sodium Fluoride required for Inhibition of Glycolysis and Respiration.

Millimols sodium fluoride per litre.	Dickens and Simer (1929).						Loebel (1925).		Lohmann (1931).		Lipmann (1928).		Ewig (1929).		Sellei & Jany (1931).	
	Jensen rat sarcoma.		Rat brain.		Rat retina.		Frog skin and brain.		Frog muscle.		Frog muscle.		Tumour.		Rat kidney.	
	G.	R.	G.	R.	G.	R.	G.	R.	G.	R.	G.	R.	G.	R.	G.	R.
100	—	—	—	—	—	—	—	—	—	—	—	—	—	—	100	64
50	100	30	—	—	—	—	—	—	—	—	—	—	—	—	—	—
15	94	0	—	—	—	—	92	—	—	—	—	—	—	—	—	—
10	91	0	86	0	85	0	—	—	—	—	100	61	100	0	67	39
5	83	0	75	0	—	—	73	35	64	—	95	27	—	—	—	—
1	50	0	33	0	—	—	57	23	37	—	50	13	—	—	0	17

G = Glycolysis. R = Respiration. The figures indicate percentage inhibitions.

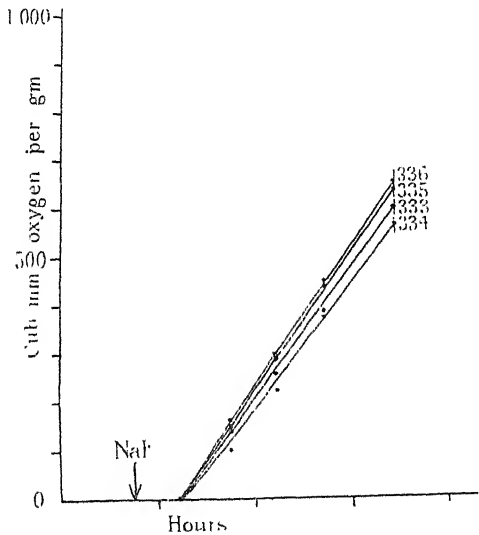


FIG. 6.—Effect of moderate concentrations of NaF (5–15 millimols per litre) on embryo respiration; experiments 333 to 336.

Higher concentrations, *i.e.*, 50 millimols and above, however, gave inhibitions of the order of 60 per cent. as shown in fig. 7, with great regularity. The relation between fluoride concentration and inhibition of respiration is plotted in fig. 8; the inhibition never becomes complete, even with very high concentrations of fluoride.

Tests were next made to determine whether there could be any regeneration of respiration after strong inhibition with fluoride (experiments 409 to 412) and it was found that by adding 5 mgm. of sodium lactate from a suspended cup some 4 hours after the commencement of the experiment, the respiration

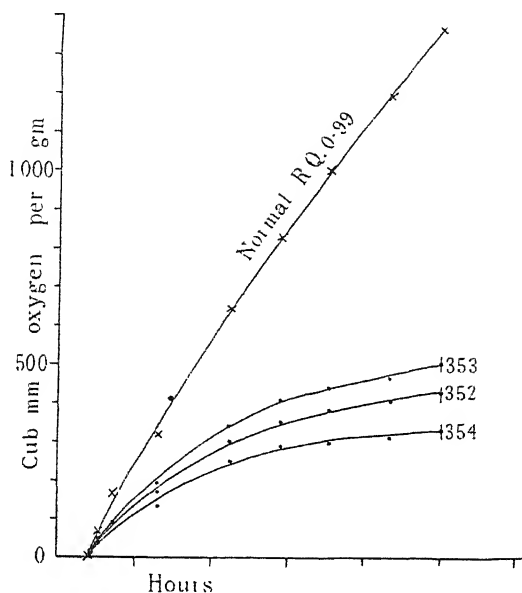


FIG. 7.—Effect of high concentration of NaF (50–75 millimols per litre) on embryo respiration.

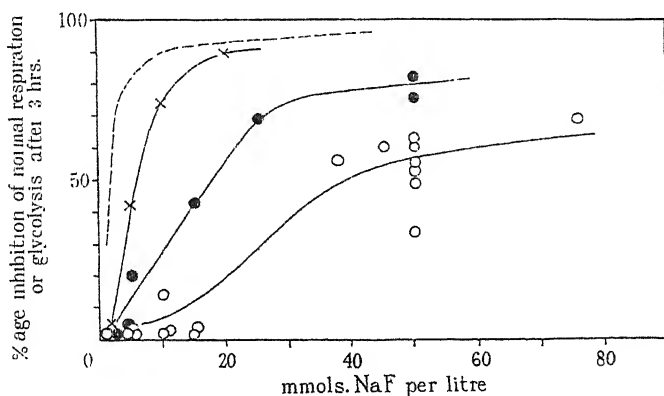


FIG. 8.—Fluoride concentration and inhibition of respiration and glycolysis.

- Respiration { ○ 4th-day embryo (experiments 329–336, 346–349, 351, 354, 431–433).
● 4th-day yolk-sac (experiments 413–416, 427–430).
- Glycolysis { × 4th-day embryo ($Q_{CO_2}^{N_2}$) (Dickens).
--- Adult rat brain (Dickens and Simer, 1929).

could be restored. It is unnecessary to reproduce details of these experiments since they give, when plotted, a picture entirely similar to that of fig. 2 (*cf.* Lipmann (1928) on adult frog muscle).

Of particular interest were the experiments on the respiratory quotient after fluoride poisoning. These are summarised in Table III. The very surprising

Table III.—Respiratory Quotients after Fluoride. Fifth-Day Embryos in Ringer-phosphate-glucose. Temperature 37° C.

Experiment No.	No. of embryos.	Wet weight mgm.		Time of experiment.	M.mols. sodium fluoride per litre.	Cub. mm. oxygen taken up.	Percentage inhibition of respiration.	Cub. mm. carbon dioxide given off.	Cub. mm. bound CO ₂ .	R.Q.
		Total.	Each.							
346	1	127	127	h. m. 3 38	5	143	5	184	30	1.08
347	1	136	136	3 38	10	146	5	165	30	0.95
351	1	162	162	4 36	10	221	5	241	25	0.98
348	2	121	60	3 38	15	143	5	175	30	1.01
349	2	183	91	3 38	50	87	60	117	30	1.00
352	2	227	113	4 36	50	96	60	112	21	0.94
353	2	177	88	4 36	50	90	60	100	21	0.90
354	2	243	121	4 36	75	81	69	115	29	1.06

result emerges that no matter how far-reaching is the inhibition of respiration, the respiratory quotient remains unity, *i.e.*, exactly the same as that of the normal embryo at this stage. Such an effect is in striking contrast to that seen with iodoacetate poisoning, where the quotient drops immediately to 0.84. It cannot be due to the utilisation of lactate originally present in the tissue, owing to the preliminary washing undergone (see the section on technique in the preceding paper of this series). It seemed that this result could only be explained in one of three ways: (1) Fluoride might have no effect at all on the glycolytic mechanism of the chick embryo, and its inhibiting effect might be solely due to a secondary action on the oxidising mechanisms. (2) Glycolysis, in the usual sense of the splitting of the hexose chain into two three-carbon chains, might play very little part in the normal carbohydrate breakdown of the embryo, some other path being in use, such as the conversion of the glucose to gluconic acid by glucose-dehydrogenase (Harrison, 1931) and the further oxidation of the latter. Ido's mysterious failure (1930) to account for the disappearing glucose as lactic acid in eggs undergoing anaerobic aseptically auto-

lysis might be relevant here. (3) The quotient of unity after fluoride might be interpreted as a protein quotient if the end-product of the process were ammonia and not urea or uric acid. Tissues *in vitro*, as Warburg (1926) showed, certainly produce ammonia if glucose is absent, and Holmes and Watchorn (1931) found that tissue-culture of embryonic rat kidney produced ammonia as well as urea in the absence of glucose or other sugars. It is not certain, however, how far these latter results can be compared with those now under discussion, since no indication of the age of the rat embryos used was given. More important, perhaps, is the fact, established by Needham (1926, *b*) that in the intact egg, ammonia excretion precedes the excretion of urea and uric acid, and reaches a maximum intensity on the fourth day of development. Protein katabolism ending in ammonia will give a respiratory quotient of about 0.95.

The first of the above alternatives is negatived for two reasons: firstly, because Dickens has shown (1932) that fluoride does inhibit the glycolysis of the chick embryo at this stage. Figures which, although unpublished, I am very kindly allowed by Dr. Frank Dickens to quote, are incorporated in fig. 8, from which it can be seen that inhibition of glycolysis at 15 millimols of fluoride is almost complete, although hardly any effect is observable at that dosage on the respiration. It is quite clear that the embryo has some additional mechanism to which it can turn for its energy. Secondly, the fact that the respiration is regenerated by added lactate indicates that a block in the lactic acid producing machinery must have been at least one of the causes of the inhibition.

In order to try whether the third alternative could be proved directly, a number of experiments were made in which the ammonia production of the embryos was measured. The results, which were conclusive enough, are summarised in Table IV.

The initial values were in exact agreement with the direct determinations of ammonia in the intact egg made some years ago. The left-hand portion of the table is a comparison between the actual oxygen-uptakes observed in each case and the ammonia produced at the same time. The latter is expressed finally as a percentage of what should have been produced if the whole of the oxygen-uptake had been due to protein combustion and ammonia-formation. In the case of normal respiration only about 20 per cent. of the respiration could be accounted for in this way, but in the case of fluoride poisoning the production of ammonia was more than sufficient to account for the whole of the respiration. The excess here was obviously partly due to the

Table IV.—Ammonia Production of Fifth-Day Embryos in the Manometer.

Experiment No.	Conditions.	No. of embryos taken.	Wet weight (mgm.).		Cub. mm. oxygen consumed per gm. wet wt.	Mgms. ammonia nitrogen produced.		Protein equivalent of oxygen consumption if all were due to protein (mgm.).
			Total.	Each.		Actual.	Per gm. wet wt.	
436	Initial	8	1051	131	—	0.023	0.022	—
437	Initial	6	960	160	—	0.018	0.019	—
							av. 0.021	
Initial value from <i>in vivo</i> experiments							0.024 (Needham, 1927)	
434	After normal respiration	3	275	91	1300	0.017	0.065	1.345
438	" "	6	895	149	1205	0.054	0.060	1.250
440	" "	5	425	85	1140	0.029	0.068	1.180
After fluoride								
435	(50 m.mols./l)	3	260	87	291	0.029	0.111	0.302
439	Do. (50 m.mols./l) ..	6	926	154	404	0.083	0.090	0.417
441	Do. (50 m.mols./l) .	5	558	111	384	0.068	0.122	0.397
442	Do. (50 m.mols./l)	5	501	100	429	0.046	0.092	0.441
After iodoacetate								
443	($0.3 \times 10^{-3}M$)	6	696	116	1125	0.050	0.072	1.170
444	Do. ($0.3 \times 10^{-3}M$) ..	6	546	91	1158	0.051	0.093	1.200
448	Do. ($0.3 \times 10^{-3}M$) ..	6	968	161	770	0.062	0.064	0.800
449	Do. ($0.9 \times 10^{-3}M$) ..	5	781	156	635	abandoned	—	—
450	Do. ($1.5 \times 10^{-3}M$) .	6	842	140	474	0.043	0.051	0.491

fact that a certain amount of respiration was proceeding before the manometer taps were turned, while the cups were coming into equilibrium with the bath, and in the right-hand portion of the table is shown an attempt to correct for this discrepancy. By extrapolating the oxygen-consumption curves on graph paper, an "additional" oxygen-uptake was found, and this, added to the previous figures, led to a still better agreement between ammonia calculated and ammonia found. Indeed, the average of the fluoride experiments worked out to 102 per cent. From these results the conclusion inevitably follows that the quotient of unity after fluoride poisoning of the embryo is due to protein combustion leading solely to ammonia formation.

In view of the fact that the ammonia might be derived from some other

Table IV—(continued).

Experi- ment No.	Protein equivalent of oxygen consumption if all were due to protein N. (mgm.).	Ammonia N. produced (minus the initial value) per gm. wet wt. (mgm.).	Percentage of the theoretical	" Additional " oxygen consumption i.e., before turning of the taps (calculated by extrapolation) (cub. mm. per gm. wet wt.).	Total plus additional oxygen consumption (cub. mm. per gm. wet wt.).	Protein equivalent of oxygen con- sumption (mgm.).	Protein N. equivalent of oxygen con- sumption (mgm.).	Percentage of the theoretical.
436	—	—	—	—	—	—	—	—
437	—	—	—	—	—	—	—	—
434	0.215	0.044	20.5	150	1450	1.500	0.240	18.3
438	0.200	0.039	19.5	270	1475	1.530	0.245	15.9
440	0.189	0.047	24.9	240	1380	1.430	0.229	20.5
								av. 18.2
435	0.048	0.090	190.0	80	371	0.385	0.061	147.0
439	0.067	0.069	117.0	200	604	0.627	0.100	69.0
441	0.064	0.101	158.0	120	504	0.522	0.084	120.0
442	0.071	0.071	100.0	160	589	0.610	0.097	74.0
								av. 102.0
443	0.187	0.051	27.2	120	1245	1.290	0.207	24.6
444	0.192	0.072	37.5	120	1278	1.320	0.212	34.0
448	0.128	0.043	33.6	130	900	0.932	0.148	29.0
449	—	—	—	130	—	—	—	—
450	0.078	0.030	38.5	130	604	0.627	0.100	30.0
								av. 29.4

source such as adenylyl pyrophosphate and have no relation to the fluoride effect, it was thought advisable to check these results by comparison with embryos poisoned with iodoacetate. As Table IV clearly shows, the ammonia production after iodoacetate poisoning was not at all sufficient to account for the respiration, although there was a slight rise above the normal. It appears that the normal respiratory quotient of the embryo at this stage is to be accounted for, as to 18.2 per cent., by protein breakdown, while after iodoacetate this figure was raised to 29.4 per cent.

It is of interest to compare this figure for the normal ammonia production of the embryo with those given by other workers for its normal lactic acid production. Warburg, Posener and Negelein (1924), Kumanomido (1928), and Dickens (1932) are in agreement that the $Q_{CO_2}^{N_2}$ of the fifth-day chick

embryo may be taken as 18, *i.e.*, that anaerobically it produces 0.072 mgm. lactic acid per milligram dry weight per hour. Now from Table IV it may be calculated that the respiratory rate of the embryo is 346 cub. mm. oxygen per gram wet weight per hour, 82 per cent. of which (the quota due to carbohydrate katabolism) is equivalent to 4.7 cub. mm. of oxygen per milligram dry weight per hour. Yet if all the lactic acid produced anaerobically were oxidised aerobically 59.8 cub. mm. of oxygen per milligram dry weight per hour would be required. We cannot, however, express this in terms of the Meyerhof quotient, for we do not know to what extent the production of lactic acid is stimulated by anaerobic conditions.

Ammonia production by the embryo is apparently not in agreement with the conclusion of Glover (1931), already mentioned, who states that he found no deamination of amino-acids *in vitro* by fifth-day chick embryos. The figures in his paper, however, do show a rise of ammonia above the controls, ranging from 12 per cent. in the case of glutamic acid to 77 per cent. in the case of proline, with an average of 33 per cent. for all the amino-acids. It would suffice to credit the fifth-day chick embryo with a moderate degree of deaminating ability.

There seems no need, then, to have recourse to the second of the three alternatives stated above.

What is perhaps a parallel case to the fluoride-inhibited chick embryo is found in the work of Ashford and Holmes (1931) on adult brain tissue. After insulin hypoglycaemia, *i.e.*, after the almost complete removal of carbohydrate from the brain, its respiratory quotient *in vitro* was still unity. This might well be due to protein katabolism ending in ammonia formation. The case of Lundsgaard's (1932) iodoacetate yeast (referred to above) may form another parallel.

(b) *On the Yolk-sac.*—A number of experiments showed that the inhibiting action of fluoride on the respiration was more quickly brought about in the case of the yolk-sac than in that of the embryo. As fig. 8 demonstrates, for a given degree of inhibition a lower concentration of fluoride is required. After inhibition by 25 millimols NaF, the respiration seemed to be regenerated by added lactate but not after 50 millimols. As for the respiratory quotient, it gave no evidence of being influenced at all by the fluoride ion, an average of 0.80 for fourth and fifth-day yolk-sac contrasting with the average 0.82 for unpoisoned material. This might lead to the conclusion that the main effect here is on the oxidative mechanisms, and that the path of breakdown and the source of energy remains the same. It is paradoxical, however, that the

Table V.—Effect of Fluoride on the Respiratory Quotient of the Yolk-sac ; Ringer-phosphate-glucose medium.

Experiment No.	Age in days.	Conditions.	Temperature °C.	Wet wt. mgm.	Time.	Cub. mm. oxygen consumed.	Cub. mm. carbon dioxide given off.	Cub. mm. bound carbon dioxide.	R.Q.
144	4	Normal .	38.4	355	h m. 2 39	305	296	43	0.84
216	5	" .	39.5	251	5 18	242	220	17	0.84
219	5	" . . .	39.5	213	5 18	257	231	32	0.75
417	4	" .	37.0	322	4 41	243	227	19	0.86
									av 0.82
418	4	1 m.mol. NaF	37.0	330	4 41	321	269	19	0.78
419	4	5 m.mol. NaF	37.0	308	4 41	248	229	19	0.85
424	5½	50 m.mol. NaF	37.0	567	2 51	150	152	31	0.81
423	5½	50 m.mol. NaF	37.0	342	2 51	78	82	22	0.76
									av. 0.80

inhibition should be more ready in the case of the yolk-sac than in the case of the embryo.

(c) *On the Blastoderm.*—The difficulties connected with accurately weighing blastoderms, referred to in the first paper of this series, make the comparisons necessitated in inhibition experiments almost impossible. Nevertheless, it appeared in experiments 282 to 294 that the respiration of blastoderms was readily inhibited with fluoride. Fig. 9 shows the results. So far as it was possible to gauge it, the inhibition seemed to be even more effective with small doses than in the case of the yolk-sac. The respiratory quotient was

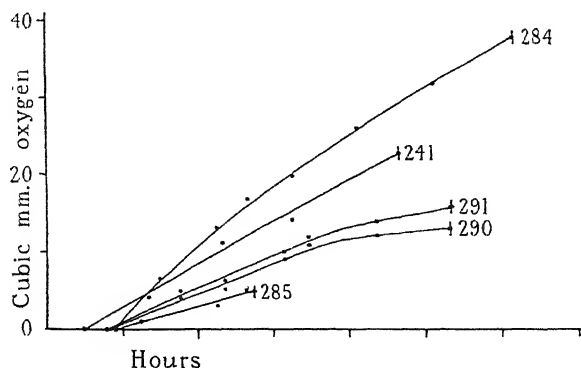


FIG. 9.—Effect of fluoride on the respiration of the blastoderm (primitive streak, no somites, 10–20 hours incubation). Experiments 241 and 284 normal, R.Q. 0.96; experiment 291, 1 m.mol. NaF, R.Q. 0.75; experiment 290, 5 m.mols. NaF, R.Q. 0.69; experiment 285, 5 m.mols. NaF.

definitely lowered to about 0.7, but it is questionable whether this implies more than the occurrence of incomplete carbohydrate combustions, for on other grounds the breakdown of fat would be unlikely. Or a selective poisoning of the *area pellucida* might explain the effect.

In order to show that it was possible to reduce the respiration of a blastoderm without reducing its respiratory quotient, experiment 298 was performed (see Table VI), in which a primitive streak was narcotised with 0.0007 M phenyl-urethane. In this case the respiratory quotient remained normal (above 0.9) although the respiration was reduced in the same way as in the experiments with fluoride shown in fig. 9.

Table VI.—Effect of Fluoride, Phenyl-urethane and Malachite Green on the Respiration of the Blastoderm (Primitive Streak Stage, no Somites); in Ringer-phosphate-glucose.

Experiment No.	Age in days.	Conditions.	Temperature °C.	Time.	Cub. mm. oxygen consumed.	Cub. mm. carbon dioxide given off.	Cub. mm. bound carbon dioxide.	R.Q.
246	0.5	Normal	39.5	h. m. 3 36	18.5	24.0	5.0	1.03
241	0.75	"	39.5	4 29	20.5	28.5	9.0	0.95
259	0.75	"	39.5	3 16	19.0	24.5	7.0	0.93
291	0.5	1 m.mol. NaF	37.0	4 54	12.5	25.5	17.0	0.69
290	0.5	5 m.mol. NaF	37.0	4 54	16.0	29.0	17.0	0.75
298	0.5	0.0007 M phenyl-urethane	37.0	4 31	25.5	34.0	11.0	0.91
305	0.5	1.0 × 10 ⁻⁴ M malachite green	37.0	3 31	16.0	18.5	12.0	0.41
306	0.5	1.0 × 10 ⁻⁴ M. malachite green	37.0	3 31	13.5	16.0	12.0	0.30

The Effect of Malachite Green on Embryo and Blastoderm.

In 1926 Yabusoe showed that numerous triphenylmethane dyestuffs, including malachite green, exercised a strongly inhibiting influence on the glycolysis of various tissues. It was of interest, therefore, to try the effect of malachite green on the respiration of embryo and blastoderm. As fig. 10 demonstrates, a marked inhibition was found with moderate doses, and the effect was not overcome by the addition of lactate late in the experiment. Inhibition must therefore have been largely due to a direct effect on the oxidising mechanisms, as would be expected from the work of Quastel and Wheatley (1931) on dyestuffs and enzymes. *B. coli* cannot oxidise lactic acid in the presence of malachite green.

Respiratory quotients were determined on the primitive streak stage, as reported in Table VI. The normal respiratory quotient for the blastoderm of approximately 0.95 was lowered to 0.4 indicating probably incomplete oxidations. Inhibition of respiration occurred just as with fifth-day embryo.

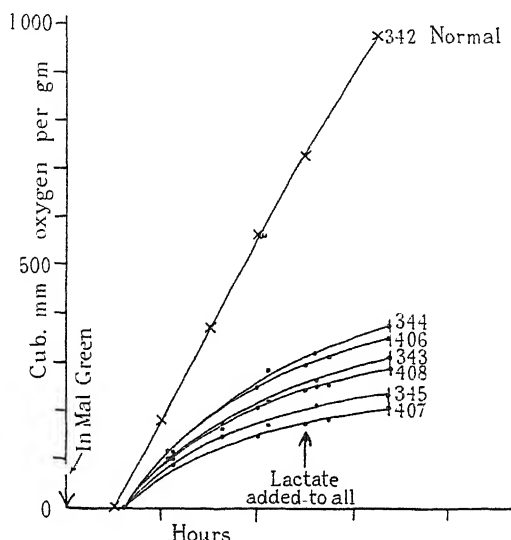


FIG. 10.—Effect of malachite green on the respiration of the embryo, 5th day. Experiments 344, 406 and 343, 1.0×10^{-4} M; experiments 408 and 345, 2.0×10^{-4} M; experiment 407, 3.0×10^{-4} M.

The Effect of Cyanide on the Blastoderm.

Speculations upon a possible anaerobic capacity of very young embryonic cells have not been uncommon in the literature of chemical embryology (see Needham, 1931, p. 742 ff., for a review of the question). For this and other reasons it was of particular interest to examine the effects of potassium cyanide upon the respiration of the chick blastoderm, in order to determine what degree of inhibition is produced by a given concentration of the poison. The only previous work on cyanide in relation to the avian blastoderm is that of Buchanan (1926), who, however, confined his investigations to the description of the morphological anomalies resulting from poisoning with HCN.

The present experiments (experiments 274 to 281) are summarised in fig. 11, from which it can be seen that after a certain point increasing concentrations of cyanide produce no further effect. These results are in complete agreement with those of Dixon and Elliott (1930, *a*), whose data for adult rabbit kidney are included in fig. 11 for comparison. But their main significance lies in the

fact that Waddington (1931), in unpublished experiments on differentiation of avian blastoderms *in vitro*, has found no effect of cyanide in concentrations below $M/1500$, as indicated upon the graph. It would thus seem as if differentiation (somite-formation, heart-formation, closure of neural tube, development of brain-vesicles, etc.) can proceed normally when the respiration is reduced

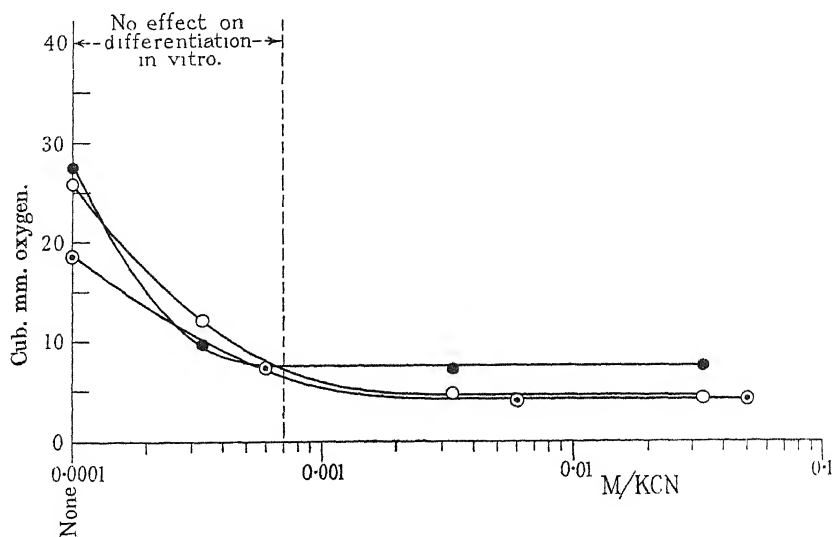


FIG. 11.—Effect of cyanide on the respiration of blastoderm (semilog plot).

- Adult rabbit kidney (results \rightarrow 10). Dixon and Elliott, p. 817 (1929).
- Chick blastoderm, 12–15 somites (experiments 274–277).
- ◐ Chick blastoderm, 10–12 somites (experiments 278–281).

to 30 per cent. of its normal value. As is well known, there is a discrepancy between the results of Dixon and Elliott on cyanide inhibition and those of Warburg (1931), who claims that the inhibition, even of the same tissues as those used by Dixon and Elliott, is complete (96 per cent.) if bicarbonate buffer is used. The question is still unsettled, and it would be very desirable to examine the cyanide inhibition of blastodermal respiration in a bicarbonate medium, but if the inhibition were complete at $0.001 M KCN$, it would only be all the more remarkable that normal differentiation can proceed up to this concentration.

The action of cyanide is, of course, upon oxidation, not glycolysis. Cyanide-inhibited blastoderms may therefore be deriving the small quantity of energy needed for differentiation from the mere desmolysis of glucose, such as that which goes on in the infertile egg (Needham, Stephenson and Needham, 1931).

Normally, as we have seen above much more lactic acid is produced anaerobically than is oxidised aerobically.

In this connection it is interesting that Lallemand (1928) found that the chick gastrula (in its quiescent state between laying and the beginning of incubation) could withstand a week's sojourn in pure hydrogen, nitrogen, and carbon monoxide, but was quickly killed by coal-gas, carbon dioxide, ammonia, and hydrogen sulphide. Immersion of frog's eggs in strong solutions of potassium cyanide has, of course, for long been used as a standard technique for freeing them from their jellies, and it would be interesting to know the effect of cyanide on their respiration. Normal development can certainly go on after such immersion when the eggs are returned to tap-water, but whether it would do so in concentrations of cyanide sufficient to reduce the respiration considerably is unknown. Brachet (1932) finds that M/1000 KCN reduces the respiration of frog's eggs by about 90 per cent.

The subject has a general importance, for if it can be shown that differentiation and respiration are independent processes during a certain period of early embryonic life, a new body of evidence will demonstrate again the remarkable facultative independence of the fundamental developmental factors (see Needham, 1931, p. 541, ff. and 1932, b).

Discussion.

The principal object of the manometric experiments recorded in this paper was the further analysis of that succession of sources of energy which seems so generally associated with embryonic development. It seemed that light might be thrown upon the transition from carbohydrate to protein by the employment of those reagents, well-known in studies on the respiration of cells, which inhibit the glycolytic process at some point or other of its mechanism. Unfortunately it is probable that neither iodoacetate nor fluoride are without secondary effects on the oxidising processes which deal with the products of glycolysis, and interpretation of inhibition experiments is thereby rendered considerably more difficult than it would otherwise be (*cf.* Ehrenfest, 1932; Lundsgaard, 1932).

If we consider the effect of iodoacetate and fluoride on the embryonic metabolism *in vitro*, we find that the two poisons differ considerably. The former inhibits the respiration while lowering the respiratory quotient, the latter inhibits but makes no change in the quotient. This remarkable constancy is certainly due to a change-over to protein katabolism ending in ammonia, but

the lowering of the quotient after iodoacetate is more difficult to understand. It might be due to protein katabolism ending in urea, although the embryo at the stage in question has not begun to form appreciable quantities of urea *in vivo*. Or it might be due to some check at a late stage in the oxidation path, leading to an accumulation of partially combusted products. Cases of this have been found by Dickens (1932), *e.g.*, a fasting liver, which gave a respiratory quotient of 0.6 and from which keto-bodies were obtained when the contents of the manometer cups was subsequently distilled. It is unlikely to be due to a participation of fat katabolism, since it has been shown (Needham 1931, p. 1171) that desaturation can only take place at a much later stage of development. One definite conclusion certainly emerges, namely, that the early chick embryo possesses the machinery for protein breakdown, and its normal (and almost exclusive) use of carbohydrate is not due to the lack of this machinery. This is exactly the opposite of the state of affairs during the second week of development, when although ample stores of carbohydrate are still available in the egg (and may be artificially increased by injection) the increase of protein katabolism follows a regular and invariable course (Needham, 1926, a). *It would seem that some as yet obscure necessity governs the succession of energy-sources, something other than the lack of the required machinery; something other than the availability of the raw materials.*

The work on the metabolism of the blastoderm from 0 to 50 hours of development, described in this paper, although valuable as the first entry into a hitherto quite unexplored region, is particularly difficult to interpret owing to the presence of two distinct and separate metabolic entities, the *area pellucida* and the *area opaca*. Progress here is hindered firstly by the difficulty of getting even an approximately accurate notion of the weights of tissue employed, and secondly by the probability that the operation of separating the blastoderm into its component parts would render it unfit for manometric work.

Particularly interesting is the contrast between the metabolic properties of embryo and yolk-sac; the former maintaining a normal respiratory quotient of unity until it becomes too big for measurement *in vitro*; the latter rapidly descending and reaching a quotient typical of fat combustion by the eighth day of incubation. Parallel with these differences goes the capacity for regeneration of respiration after the inhibition of glycolysis when lactate is added.

It is hoped to continue the line of work here described, in the following ways. Urea estimations on embryos after iodoacetate poisoning should differentiate between the possible explanations for the respiratory quotient of 0.82 then

found. The middle and final periods of the yolk-sac will be examined as regards their normal respiratory quotient and their behaviour towards glycolysis-inhibiting reagents, while so far the allantoic membrane has been altogether untouched, and its metabolism is entirely uncharted. The extraction of enzymes from the early embryo, moreover, would afford valuable information, and the behaviour of its soluble lactic-acid enzyme, for instance, would well repay investigation. Again, can glucose or hexose phosphate best be utilised by the embryo during its carbohydrate phase? The answer to this question would be of great interest from the point of view of the succession of energy-sources, for if glucose could be oxidised by the embryo without phosphorylation, as Bumm and Fehrenbach (1930, 1931) have suggested is possible in some adult tissues, some light might be thrown on the problem of why the embryo begins with carbohydrate katabolism instead of with protein or with fat. Finally, the interesting work of Barron (1929, 1930) and Barron and Hoffmann (1930) on marine eggs with dyes in relation to cell-respiration, and similar observations of Chang and Gerard (1931) on nerve cells, suggests that this method might also be applied to the early stages of avian development.

The author is much indebted to Dr. Dorothy Needham for her collaboration in some of the experiments. He would also acknowledge the kindness of Dr. Frank Dickens, Mr. C. H. Waddington and M. Jean Brachet in allowing him to make reference to their unpublished observations. The Government Grant Committee of the Royal Society furnished a grant which partially defrayed the cost of the work.

Summary.

(1) The effects of iodoacetate, fluoride, cyanide, phenyl-urethane, and malachite green upon the respiration and respiratory quotient of the chick embryo, yolk-sac, and blastoderm *in vitro* during the first week of incubation have been studied in an attempt to delineate the metabolic possibilities of the developing cells. Ringer-phosphate-glucose medium at p_H 7.4 was used throughout.

(2) Iodoacetate in moderate doses inhibits the respiration of the embryo after a certain latent period, but the effect is reversible, for oxygen-consumption recommences if lactate is added. The respiratory quotient drops from 1.0 to 0.84 as soon as the embryo is treated with iodoacetate. These facts are interpreted as implying (a) a rapid action of the drug on some part of the oxidation mechanism, leading to the incomplete oxidation of carbohydrate, (b) a delayed action on glycolysis, suppressing the supply of lactic acid.

(3) Iodoacetate in moderate doses inhibits the respiration of the yolk-sac, but not quite so completely as in the case of the embryo. The inhibition is irreversible; it cannot be removed by addition of lactate. This is in agreement with the generalisation that only tissues with a normal respiratory quotient of unity can have their respiration restored by lactate after iodoacetate poisoning (Dickens).

(4) Iodoacetate also inhibits the respiration of the blastoderm, but regeneration by lactate is variable, probably depending on the relative activity of *area opaca* and *area pellucida*.

(5) Fluoride in doses sufficient to abolish glycolysis completely has no effect on the respiration of the embryo, which is only inhibited by much higher concentrations. This inhibition is reversible, like that of iodoacetate. But no matter how far-reaching the inhibition of respiration, the respiratory quotient remains at its normal level, *i.e.*, unity. This has been quantitatively shown to be due to protein katabolism having ammonia as its end-product.

(6) Protein combustion of this character has also been shown to be responsible for about 20 per cent. of the normal respiration of the embryo (R.Q. 1.0), and this proportion is not significantly changed after iodoacetic acid poisoning.

(7) Fluoride inhibits the respiratory activity of the yolk-sac in relatively lower concentrations than in the case of the embryo. It was not found to have any effect on the respiratory quotient, which remained at its normal level (0.82).

(8) Fluoride also inhibits the respiration of the blastoderm and lowers its respiratory quotient. As the blastoderm is not a homogeneous entity, this lowering is difficult to interpret.

(9) The respiration of a blastoderm may, however, be partially inhibited, without any effect on the respiratory quotient, as is shown by a narcotisation experiment with phenyl-urethane.

(10) Malachite green, in doses sufficient to inhibit glycolysis, inhibits the respiration of the embryo and blastoderm completely after a certain time, and the oxygen-uptake cannot be restored by addition of lactate. The respiratory quotient is very much lowered.

(11) Cyanide strongly inhibits the respiration of 10- to 15-somite blastoderms, and the relation between concentration of cyanide and percentage inhibition is quite similar to that established for many adult tissues by other investigators. Yet the differentiation of the blastoderm *in vitro* (Waddington) is unaffected by concentrations of cyanide at which at least 70 per cent. of the normal respiration is abolished.

(12) It is concluded in general that the preferential katabolism of carbohydrate by the embryo in the first week of its development is not due to any lack of the machinery necessary for katabolising protein, for this can take place if carbohydrate breakdown is experimentally suppressed.

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Studies on the Hypophysectomised Ferret. I.—Technique.

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[PLATE 2.]

I. *Introduction.*

The difficulty of performing hypophysectomy as an operation has greatly retarded the study of the relation between the anterior lobe and the gonads. Practically all our knowledge of the effects of hypophysectomy on the reproductive organs has arisen from Smith's great work on the rat (summarised in 1930).^{*} Several workers have followed Smith on the rat, and the operation has more recently been performed on the cat (Allan and Wiles, 1932), the monkey (Hartman, Firor and Geiling, 1930), and the rabbit (Smith and White, 1931). In the last two animals the operation is one of considerable difficulty, due to the inaccessibility of the hypophysis. The ease with which the operation can be performed in any given species depends largely on the anatomy of the skull and neck. In this connection the shape of its head suggested that

^{*} No attempt is made here to summarise the literature relating to hypophysectomy. Much of the work, especially that carried out on dogs, has had a purely clinical objective and is not of great significance from our present point of view.

the ferret might be highly suitable, an idea confirmed by examination of the skull and dissection of the appropriate region of the neck. Tentative experiments showed that the operation was quite feasible on the ferret and there is little doubt that this species offers the easiest hypophysectomy yet described.

The normal reproductive processes of the ferret are well known (Marshall, 1904; Hammond and Marshall, 1930) and need not be described here, except to say that they are such as to make the animal highly suitable for experimental laboratory work. The advantages of being able to hypophysectomise an animal in which there is a good external sign of œstrus and in which the time of ovulation can be calculated to within a few hours are obvious.

II. *Surgical Technique.*

The animal is given atropine, and anaesthetised with ether. It is fixed in the dorsal position, the neck being stretched out by an elastic band through the mouth. A tracheal cannula is then inserted and further anaesthetic administered by this route. A tracheotomy, while not imperative, is highly desirable owing to the fact that the pharynx is completely occluded during the later stages of the operation by the pressure of the retractor. We have performed the operation without a tracheotomy, but work has to be suspended at frequent intervals to allow the animal to breathe.

Another incision, about 2 inches long, is made, beginning about half an inch from the tip of the lower jaw, and passing backwards and slightly obliquely, to the left of the mid-line. The anterior limit of the wound is marked by the point of emergence of the left lingual vein through the mylo-hyoid muscle. The sub-maxillary gland, circular in outline and about 1 cm. in diameter, is situated just behind and below the angle of the jaw, and immediately in front of it a transverse vein passes across the digastric muscle into the anterior facial vein. This vein, which is not always prominent, and which was indeed absent in three cases out of 32, is divided between ligatures at the medial border of the digastric muscle, and the submaxillary gland is retracted laterally.

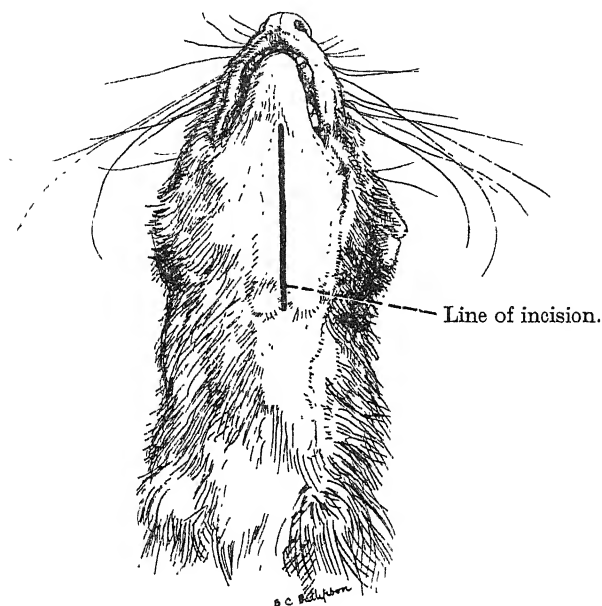
The region just in front of the submaxillary gland is then dissected. The hypoglossal nerve is exposed, and also the stylo-glossus muscle covering the epihyoid bone. The nerve is dissected backwards until it is seen crossing the external carotid artery. The next step is to divide the stylo-glossus muscle and expose the epihyal, which is removed with scissors. When this is done the bulla is revealed, covered by fascia and muscular fibres. The surface of the basi-sphenoid is reached by breaking through the covering of fascia and

periosteum at its junction with the bulla, and by gently pushing the periosteum and its attached structures in a medial direction. Bleeding at the angle of the bulla always occurs at this stage, but is readily stopped by applying a cautery. The basi-sphenoid is further exposed by pushing with swabs; the pharynx meanwhile being kept to the right of the mid-line by a curved retractor. The floor of the pituitary fossa is then seen as a pink circular area about 2 mm. in diameter, just about level with the anterior end of the bulla. A number 16 dental burr, driven by a foot drill, is applied just behind the centre of this pink area, and the bone drilled through to produce a hole about 3 mm. in diameter. The bone here is surprisingly avascular, and marked oozing indicates that the burr has been applied too far back. When the dura below the drilled area is cut, a little blood (from the small venous sinuses in that area) and some cerebro-spinal fluid leaks through, and the pituitary gland bulges into the opening. It is removed by the application of a glass suction pipette (about 2 mm. in diameter). No attempt is usually made to scrape out the pituitary fossa with an instrument, but a smaller pipette is used to remove fragments of pituitary tissue from the periphery of the fossa. The stalk is broken off as near as possible to the diaphragma sellæ, and usually little or no bleeding occurs at this stage. The operation area is finally washed with saline, the superficial muscle stitched and the wound closed. In two of the earlier operations, the hole through the basi-sphenoid was plugged with wax after the removal of the pituitary body. There seems, however, to be no advantage in doing this.

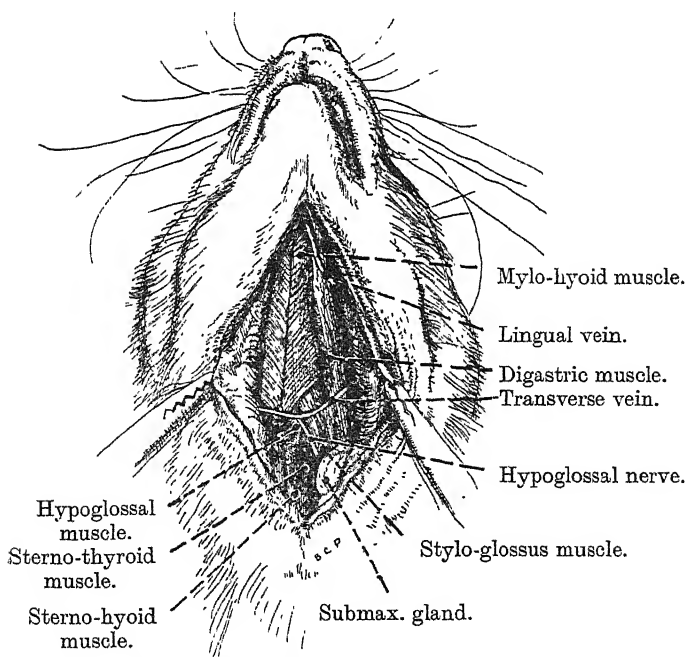
Certain precautions are necessary in removing the tracheotomy tube and closing the trachea. In spite of the atropine, much mucus often collects in the throat during the operation, and it is desirable to swab this out so far as possible before removing the tube. In addition, seepage from the damaged tissues may have entered the upper part of the trachea through the tracheotomy opening and should be removed by a small swab. Before closing the hole, we have found it useful to allow the animal to recover partially from the hitherto deep anæsthesia in order that the respiration may be sufficiently vigorous to clear the throat. One stitch is usually enough to close the trachea. Finally, care should be taken to avoid seepage from the tissues surrounding the trachea after the wound is closed. With these precautions the tracheotomy seems to have no adverse effect on recovery.

The time occupied by the sterile operation is between 1 to 1½ hours, of which about one-quarter is taken up after removal of the pituitary body. The shortest time, allowing for the preparation of the animal, in which the pituitary body

can be removed after a given time (such as the end of copulation) is about 1 hour.



(a) Line of skin incision.



(b) Superficial relations after section of the skin and platysma.

FIG. 1.—Stages of the operation of hypophysectomy in the ferret.

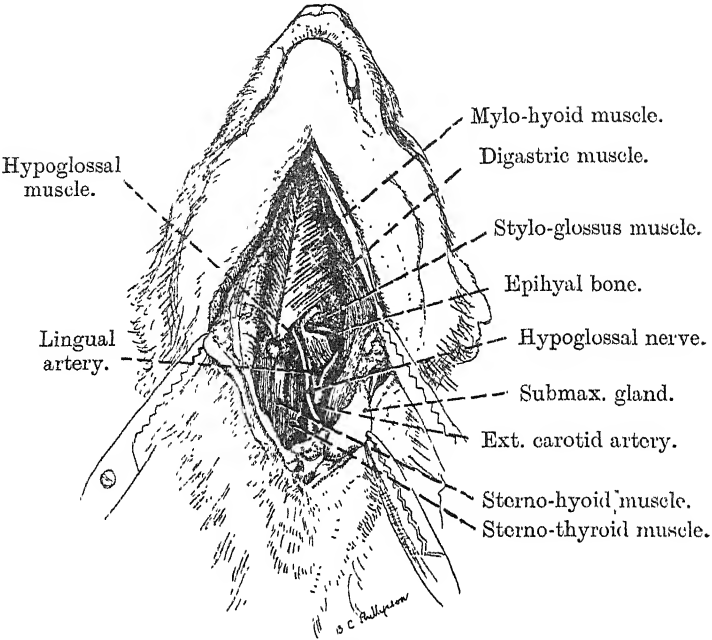
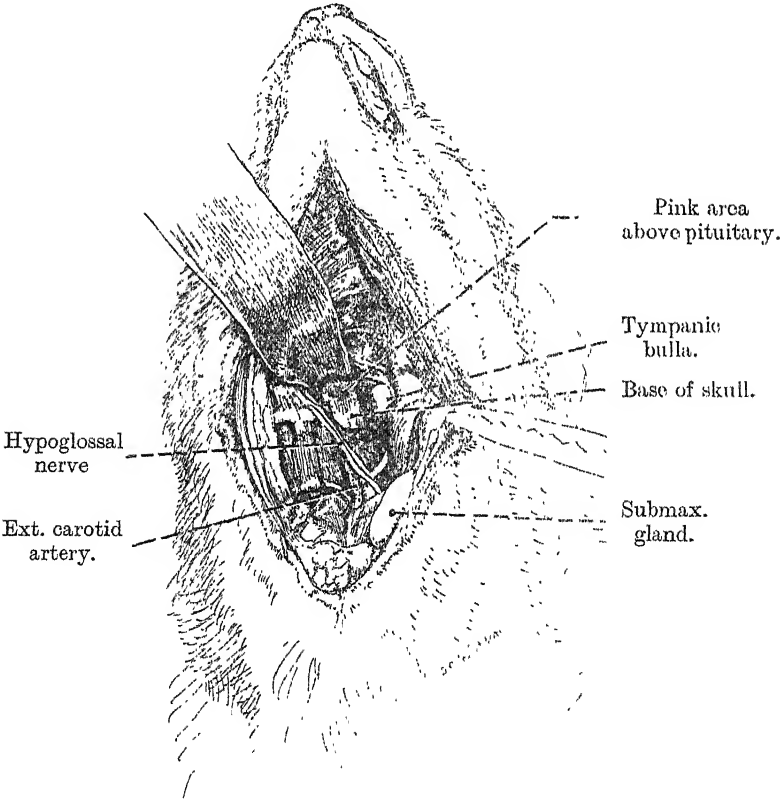
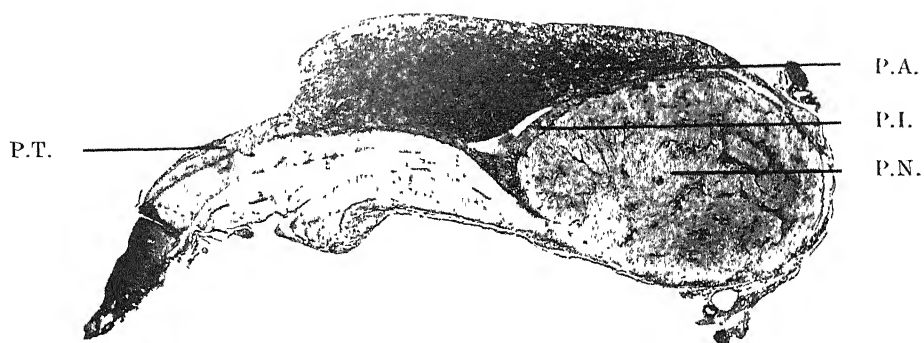
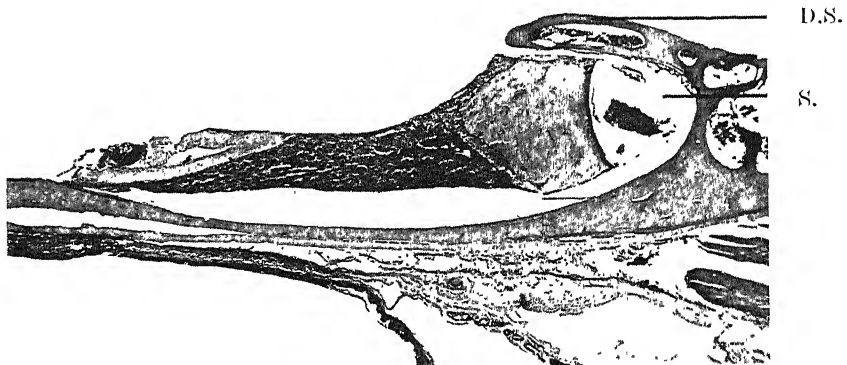


FIG. 1.—(c) Exposure of the hyoid bone.

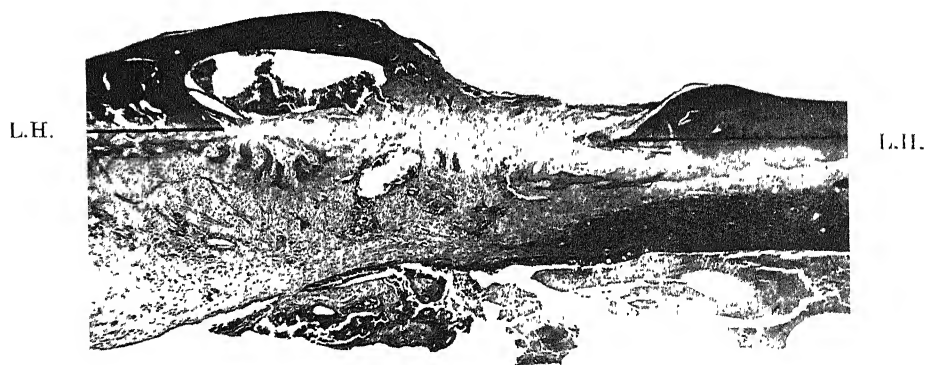




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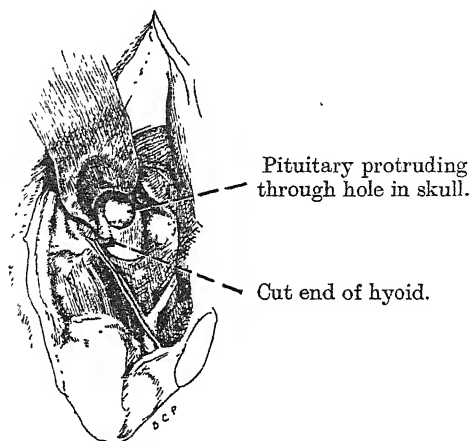


FIG. 1. —(e) Exposure of the pituitary after drilling the basi-sphenoid.

III. Recovery.

The animals are usually quite round within 1 to 2 hours after the end of the operation. They drink readily soon afterwards. Water only is given until next day, when milk is substituted. Within 2 days of the operation meat is eaten if cut up into pieces which can be swallowed whole. After another 2 days, most of the animals are willing to chew large pieces of meat, and subsequent recovery is uneventful. Many of our animals have had a gonadectomy performed within a few days of the hypophysectomy without adverse effects. Excluding two anæsthetic deaths on the table and one animal dying rapidly owing to damage to the cavernous sinus, we have lost only one operated case. This died, for reasons unknown, on the day following the operation. Most of the animals have been killed in apparently good health at definite times to obtain stages of gonad degeneration. The longest they have so far been allowed to live is 4 months, at which time the animal is indistinguishable from a normal, except for being somewhat lethargic. The chronic snuffle noted by other workers following parapharyngeal hypophysectomy has been little in evidence in the ferret.

IV. Post-mortem Findings.

At autopsy, routine examination was made of the pituitary site as follows. The head was cut from the animal to facilitate drainage of blood, and the top of the skull sawn off. The brain was then removed from within the dura in the usual way. In the majority of cases the base of the brain was adherent

to the diaphragma sellæ at the site of the remains of the infundibulum. The degree of adherence varied in different animals, but was usually over an area of about 1 mm. square. The firmness of the adhesion depended on the time after operation. Following removal of the brain the diaphragma sellæ was seen covering the hole in the basi-sphenoid, the area formerly raised being now depressed. In some cases the remains of the stalk could be seen as a small nodule embedded in the diaphragma. In one case (the second sterile operation) an obvious piece of pituitary tissue was seen lying on the left side of the cavity. In no other animal was there any macroscopic sign of pituitary tissue.

In animals used for specific experiments on the reproductive organs it was clearly desirable to make a more thorough examination of the pituitary fossa. The base of the skull was therefore fixed, decalcified and serially sectioned. A typical median longitudinal section is given in fig. 4, Plate 2, and shows the local effects of the operation in an animal killed 28 days later. In this section, the basi-sphenoid has been removed for about two-thirds of the distance underneath the dorsum sellæ, and for a rather greater distance in front. The dorsum sellæ has collapsed, and the posterior portion of the sella is occluded.

Of the first 13 hypophysectomised animals examined microscopically, the second and the fourth (MHF 2 and 5) had pieces of tissue which were obviously anterior lobe, while five others had a fragment of tissue which may possibly have been of hypophyseal origin. So far as could be determined, the remaining animals were definitely devoid of pituitary tissue. Only MHF 2 and 5 showed any physiological signs of having active anterior lobe tissue.

V. *Discussion.*

Comparing the operation with that described for other laboratory animals it becomes obvious that the ferret has numerous advantages. Among the most important of these are :—

- (a) That no very important structure is removed or destroyed on the way to the base of the skull.
- (b) That every stage of the operation is plainly visible.

The removal of the epihyoid does not appear to inconvenience the animals. In two operations there was a suspicion that the pharynx had been pierced, but in any case recovery was uneventful.

The thin floor of the pituitary fossa, with its pink colour due to the gland, forms an invaluable landmark in this operation. It leads the burr directly

to the centre of the fossa, and between the cavernous sinuses, which thus escape injury. The main venous cross-channel connecting the cavernous sinuses in the area of the pituitary fossa passes just anterior to the dorsum sellæ and posterior clinoid processes, and it too escapes injury. The gland does not reach to the posterior limit of the fossa, only a fraction being under cover of the dorsum sellæ, fig. 3, Plate 2. This portion is composed almost exclusively of pars nervosa and pars intermedia, the pars anterior, with which we are mostly concerned in work on the reproductive processes, being much more accessible. So far as can be determined the pars tuberalis appears to cover the surface of the stalk only as far as the point of emergence through the diaphragma sellæ, fig. 2, Plate 2, and is thus removed with that portion of the stalk.

It is a pleasure to acknowledge our obligation to Dr. S. Zuckerman, who studied the anatomy of the ferret's neck for the purpose of describing the operation and who supervised the preparation of fig. 1. We are also indebted to Mr. H. Allan and Mr. P. Wiles for showing us their operation on the cat, and to Miss M. Allanson for sections of the ferret pituitary. Fig. 1 is by Miss B. C. Phillipson.

VI. *Summary.*

A technique for parapharyngeal hypophysectomy in the ferret is described.

DESCRIPTION OF PLATE.

Guide Letters.—D.S., Dorsum sellæ; L.H., Limit of drill hole; P.A., Pars anterior; P.I., Pars intermedia; P.N., Pars nervosa; P.T., Pars tuberalis; S., Sinus.

FIG. 2.—Longitudinal section of pituitary body of normal male ferret, showing extent of pars tuberalis. $\times 30$.

FIG. 3.—Longitudinal section through base of skull of normal male ferret, showing position of the hypophysis in the sella turcica. $\times 20$.

FIG. 4.—Longitudinal section through base of skull of ferret 30 days after hypophysectomy. This section is almost through the centre of the drill hole, and shows almost its greatest longitudinal extent. $\times 20$.

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Studies on the Hypophysectomised Ferret. II.—Spermatogenesis.

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[PLATE 3.]

I. Introduction.

Smith (1930) has shown that all parts of the reproductive tract of the male rat show pronounced atrophy after hypophysectomy. The testes are much reduced in size and are flabby. The seminiferous tubules show a corresponding diminution in size and all indications of spermatogenesis are absent. A more immediate effect of ablation of the pituitary gland is a complete loss of interest in the female. Richter and Wislocki (1930) also noted atrophy of the male genital organs of the rat after hypophysectomy, but in no great detail.

Hypophysectomy has not yet been performed on a species in which the male shows a clear cut ancestrous period such as is found in the male ferret (Allanson, 1932). The present work was undertaken to find out if the testes of the ferret react to hypophysectomy in the same way as those of the rat, and to compare the resulting condition of the testes with that found during ancestrus. Further light on the activity of the pituitary body during ancestrus might thus be obtained. In addition, it was hoped to determine the rate of regression after hypophysectomy and to compare this with that found at the end of the breeding season. No attempt will be made in this or the following paper to deal with the general effects of hypophysectomy, but it may be mentioned that loss of body weight, if any, was slight during the time covered by these experiments.

II. Methods and Technique.

Six mature male ferrets in the breeding season (June) were selected for experiment. Hypophysectomy was carried out on five of these animals by the method described in Part I, the remaining ferret being kept as a control and killed in the first week of August, 22 days after the experimental animals had been killed. For histological study the right testis and epididymis were removed from each ferret at various times after hypophysectomy. The

animals were killed 14 days after the unilateral castrations. In this way two stages of the post-hypophysectomy changes in the testis were obtained from each animal.

All the material was fixed in Bouin's fluid (alcoholic) immediately on removal from the animal, cut at 10 μ and stained with Meyer's hæmalum and eosin in the usual way. For comparison of the size of the seminiferous tubules, the average diameter of 10 tubules in each testis was taken. The diameter of each tubule was found from camera lucida drawings by taking the largest measurement at right angles to the longitudinal axis.

At autopsy, careful examination for the completeness of the removal of the pituitary was made, as described in Part I.

III. *Experimental Results.*

Completeness of removal of pituitary tissue.—Following Smith's statement that one of the immediate results of hypophysectomy is complete loss of libido, the ferrets were put with females soon after the operation. MHF 3 and 5 appeared to be in rut, but MHF 2, 4 and 6 showed no specific interest. Before killing, each ferret was again placed with a female; MHF 3 copulated, but not MHF 5, and the remaining animals again exhibited no interest. On histological examination of the pituitary fossæ, MHF 3 was found to have a large piece of anterior pituitary tissue and MHF 5 a smaller piece. As will be seen from Table I this amount of pituitary gland is apparently quite sufficient to maintain the reproductive organs in a practically normal condition, and therefore MHF 3 and MHF 5 must be placed in a different category to the remaining animals. MHF 2 appeared to be entirely devoid of anterior pituitary tissue. In MHF 4 and 6 fragments were found which were almost certainly remains of anterior lobe. From the fact, however, that the testes obtained from these animals fall into series with those obtained from MHF 2, it is safe to conclude that these fragments were not significantly functional.

Gross changes in the testis.—Less than a fortnight after hypophysectomy there was a noticeable diminution in size of the testes, which became less easy to palpate. Later, at 21 days, they were withdrawing from the scrotum. At 30 days after hypophysectomy when MHF 2 was killed, the left testis was found in the inguinal canal.

Table I and fig. 1 show the decline in weight of the testes from 7 days to 30 days after hypophysectomy. The time between right and left castration was 14 days in each animal; the greatest percentage decrease in weight during

this time was 54 per cent., during the earliest period after hypophysectomy. This fact, together with the slope of the curve in fig. 1, shows that the rapidity of weight decrease is greatest in the early stages of degeneration.

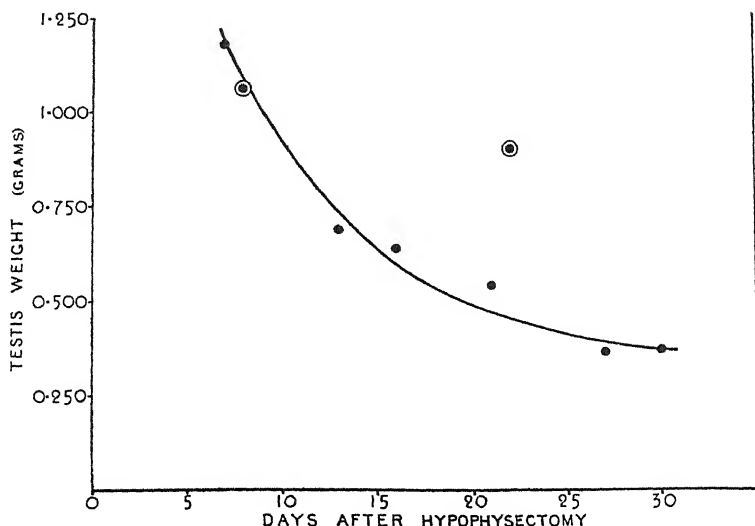


FIG. 1.—Weight of the testis after hypophysectomy.*

Gross changes in the epididymis.—As will be seen from Table I the decrease in testis size is accompanied by diminution in the weight of the epididymis, which, plotted against the testis weight, gives an exactly similar curve to that found by Allanson (1932) for the normal ferret. It may be concluded that the regression of the testis is paralleled by an accompanying regression of the epididymis, and that the latter does not lag further behind than is usual at the end of the breeding season.

Histology of the testis.—With the diminution in weight of the testis there is a corresponding decrease in the size of the seminiferous tubules (fig. 2). Allanson (1932) gives the diameter of the tubule of the normal ferret from May to August as 140–180 μ . The corresponding figure for our control ferret killed early in August is 185 μ . The average diameter of the tubules of the experimental animals decreases from 172 μ at 7 days after hypophysectomy to 90–92 μ at 27–30 days after. These changes in the size of the tubule are correlated with decrease in the activity of the seminiferous epithelium. At 7 days after hypophysectomy, fig. 4, Plate 3, there is little abnormality in

* In this and the following graph the points in circles relate to incompletely hypophysectomised animals.

Table I.

Number of animal.	Date of hypo-physectomy.	Right testis.			Time of autopsy after hypo-physectomy (days).	Left testis.			Completeness of hypophysectomy.
		Time of removal after hypo-physectomy (days).	Weight (gm.).	Average diameter 10 tubules (μ).		Weight (gm.).	Average diameter 10 tubules (μ).	Left epididymis weight (gm.).	
MHF 2	14.6.32	16	0.639	142	30	0.368	92	0.116	Apparently complete.
MHF 3	16.6.32	14	1.473	187	28	1.910	195	0.303	Piece present.
MHF 4	17.6.32	13	0.687	142	27	0.364	90	0.113	? Fragment.
MHF 5	22.6.32	8	1.060	187	22	0.898	157	0.170	Small piece present.
MHF 6	23.6.32	7	1.179	172	21	0.538	127	0.187	? Fragment.
MHF 7	(Control)	—	0.956	185	Aug. 5th	0.957	185	0.229	—

the state of the testis. Mature spermatozoa are present in numerous tubules, while in others advanced spermatids, elongating to form spermatozoa, are found. In certain tubules there is clumping of spermatids and spermatocytes in the lumen, but this condition is not sufficiently frequent to indicate regression.

At 13 days after hypophysectomy, fig. 5, Plate 3, there are definite signs of degeneration of the seminiferous tubules. No spermatozoa are present, and though some tubules still have three or four regular layers of spermatids,

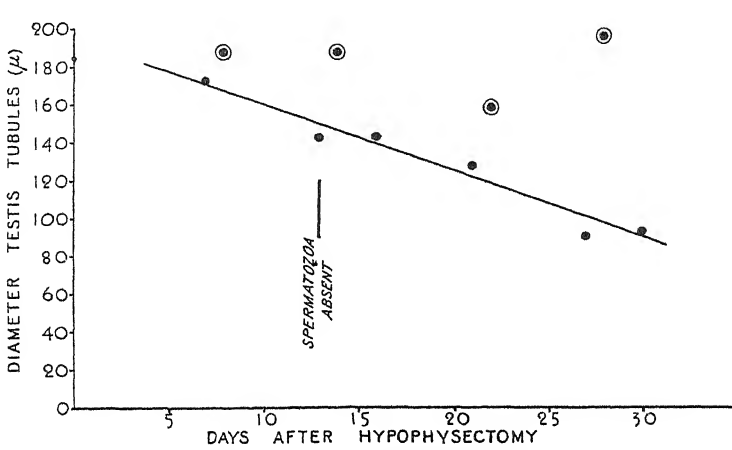


FIG. 2.—Diameter of testis tubule after hypophysectomy.

many are filled with loose masses of degenerating spermatocytes and spermatids, or clumps of spermatids. Others again contain large cytoplasmic masses, enclosing spermatid nuclei, which obviously indicate some interruption of the normal process of spermatogenesis.

At 16 days after hypophysectomy the tubules have shrunk considerably. A few are lined by two layers of cells only, spermatogonia and Sertoli cells with spermatocytes. Loose masses of degenerating spermatocytes and spermatids are found as in the 13-day stage. While the production of spermatocytes is still going on, it would appear that, when they develop into secondary spermatocytes and spermatids, sloughing takes place to form degenerate masses in the lumen. In some tubules are found what appear to be the crumpled remains of degenerating spermatozoa.

At 21 days after hypophysectomy, the tubules appear more uniformly atrophic than in the 16-day stage. Though most of the tubules have primary spermatocytes, occasionally one may be found consisting only of a single layer

of spermatogonia and Sertoli cells: degenerating cell masses are present as before in the lumen.

At 27 days after hypophysectomy there are many more tubules than in the 21-day stage consisting of one layer only of spermatogonia and Sertoli cells. Other tubules still contain primary spermatocytes forming secondary spermatocytes but probably not spermatids. In a few tubules the remains of spermatozoa persist abnormally long. The whole testis gives the appearance of considerable regression and very little activity.

At 30 days after hypophysectomy, fig. 6, Plate 3, the tubules are completely atrophic, the majority consisting of one layer of spermatogonia and Sertoli cells. Occasionally tubules containing primary spermatocytes are present but no later stages of spermatogenesis are detectable. Sometimes the spermatocytes are scattered in the lumen and appear degenerate, but no other cell masses are seen. The whole appearance is that of the normal testis at its lowest ancestral state in November. No attempt has been made here to study the condition of the interstitial cells, but it may be noted that in this last stage the cytoplasm of the cells is still extensive but very vacuolated, indicating the presence of fat, while in the normal ancestral animal the cells are small, the nucleus fills the whole cell, and very little fat is present.

Histology of the epididymis.—The histological changes in the epididymis during the month following hypophysectomy show a gradual approach to the ancestral condition. At 30 days after removal of the pituitary (MHF 2) the epididymis presents the typical appearance of ancestrus. The tubule is shrunk and the muscular coat is thickened, while the epithelium is low and has no cilia. In one important particular, however, the rapidly atrophied epididymis of the hypophysectomised animal differs from the more slowly atrophied one of the ancestral animal, *i.e.*, in the presence of spermatozoa. All epididymes up to 16 days after hypophysectomy contain an abundance of spermatozoa. At 21 days after, a number of spermatozoa are found, while at 27 and 30 days they are still present, though in small numbers. This presence of spermatozoa in conjunction (after 13 days) with atrophic testes and epididymes is undoubtedly due to the rapid degeneration of the latter, since Moore (1928) found that the longevity of spermatozoa in the epididymis of the castrated guinea-pig is up to 23 days, while histological integrity persists even longer.

We are indebted to Miss M. Allanson for assistance with the comparison of the normal and experimental animals.

IV. *Summary.*

(1) Hypophysectomy of the male ferret during the breeding season (June) causes regression of the testes to the November anæstrous condition within about a month.

(2) The regression is characterised by decrease of testis weight and by aspermatogenesis.

(3) The experimental regression, though leading to exactly the same condition, is about three times as rapid as the normal decline into anæstrous.

(4) The epididymis shows similar atrophy.

DESCRIPTION OF PLATE.

Testis. $\times 120$.

FIG. 3.—Testis of normal ferret killed on August 5. Activity just beginning to wane.

FIG. 4.—Testis of ferret (June 30) 7 days after hypophysectomy. Few spermatozoa.

FIG. 5.—Testis of ferret (June 30) 13 days after hypophysectomy. Aspermatic and degenerating.

FIG. 6.—Testis of ferret (July 14) 30 days after hypophysectomy. Complete anæstrous condition.

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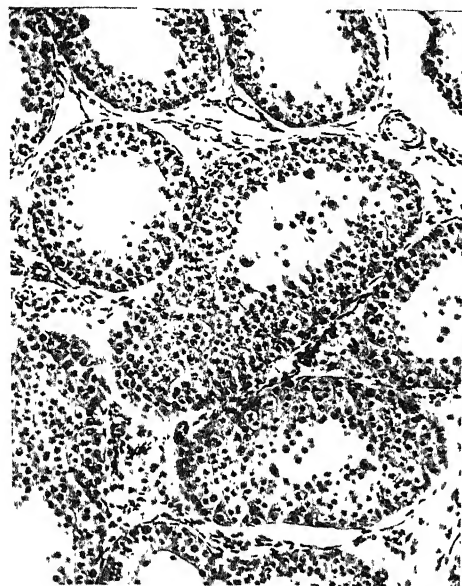
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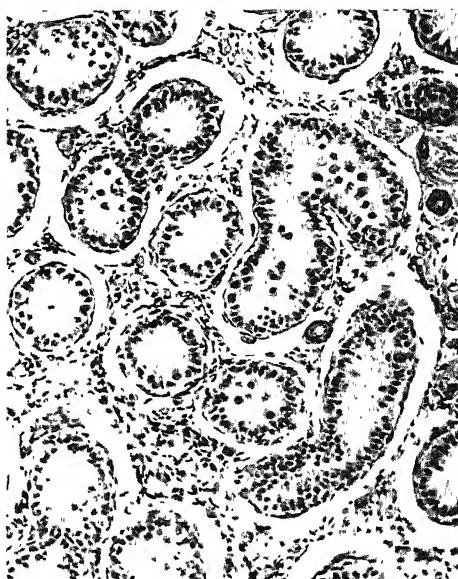
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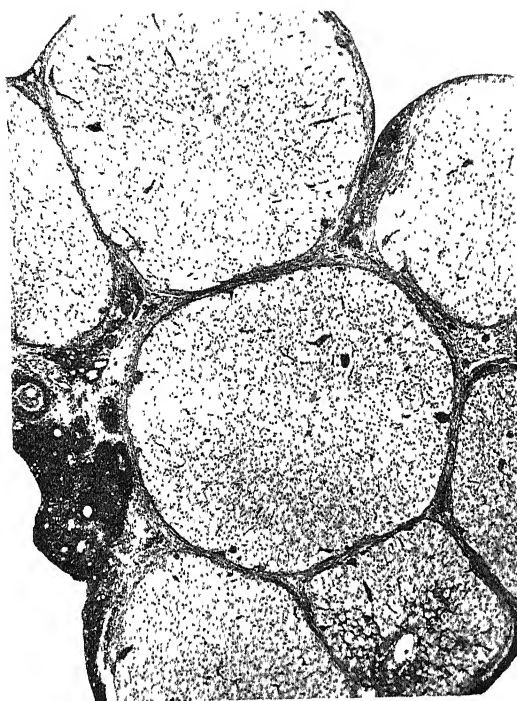
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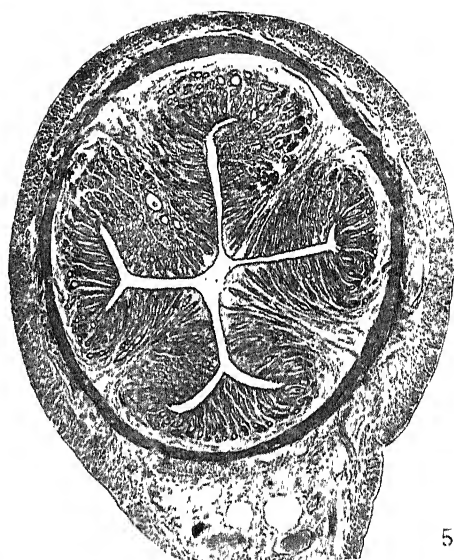
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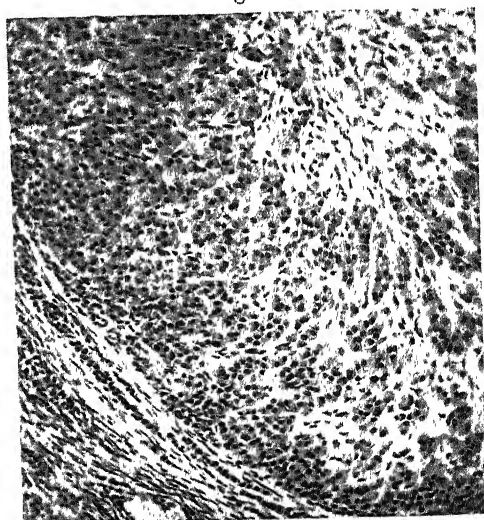
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6

Studies on the Hypophysectomised Ferret. III.—Effect of post-coitus hypophysectomy on ovulation and the development of the corpus luteum.

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[PLATE 4.]

I. *Introduction.*

In animals which ovulate only after copulation it is necessary to suppose that nervous stimulation of the pituitary body results in the liberation of the ovulation-producing substance. Fee and Parkes (1929) were able to show that in the rabbit removal of the pituitary body (by decerebration) later than 1 hour after copulation did not prevent the ensuing ovulation; removal earlier had an inhibitory effect, which could, however, be overcome by injection of anterior lobe substance (Hill and Parkes, 1931). These results indicated that following copulation, the ovulation-producing hormone was secreted into the circulation with great rapidity. In addition, the removal of the pituitary body before ovulation was found not to derange the initial development of the corpus luteum so far as this could be followed on decerebrate preparations (Deanesly, Fee and Parkes, 1930).

Smith and White (1931) confirmed these results on rabbits hypophysectomised as an operation. They were not able to complete the removal sufficiently soon after copulation to inhibit ovulation, but they extended the work to a more prolonged study of the development of the corpus luteum. The growth of this body in the hypophysectomised rabbit was normal for about 2 days, but afterwards became atypical.

The technique described in Part I of the present series has enabled us to extend this type of work to the other mammal definitely known to ovulate only after copulation—the ferret. This animal has the advantage over the rabbit that the time between copulation and ovulation is rather more than three times as long, *i.e.*, about 36 hours, and that pseudo-pregnancy, during which the life-history of the corpus luteum may be studied, lasts about 6 weeks (Hammond and Marshall, 1930). On the other hand, the fact that copulation takes at least three-quarters of an hour makes it difficult to remove the pituitary

within less than about $1\frac{3}{4}$ hours of the commencement of the act. No attempt will be made here to describe the ultimate effects of hypophysectomy on the ferret ovary.

II. *Material and Technique.*

The hypophysectomy technique has been described in Part I, together with the method of examination at autopsy. In the animals dealt with in the present paper, sections through the base of the skull showed no tissue which could positively be identified as hypophyseal, though one ferret (RHF 15) had two small pieces of tissue which were probably remains of anterior lobe, and two other animals each had a minute fragment which may possibly have been of pituitary origin. The remaining five ferrets so far as could be seen were completely devoid of pituitary tissue.

Two stages in the post-hypophysectomy changes in the ovaries have been obtained from most of the animals by performing a unilateral ovariectomy soon after ovulation should have taken place, and afterwards killing the animal during what would have been the ensuing pregnancy. In some cases where the ruptured follicles were abnormal serial sections of the Fallopian tube were cut. The presence of tubal ova was taken as a definite indication that ovulation had occurred.

The usual histological technique was employed.

III. *Effect on Ovulation.*

Table I shows the effect on ovulation of removal of the pituitary gland up to 15 hours post-coitum. It is immediately apparent that ovulation is not inhibited by even the earliest ablation achieved—1 hour 50 minutes after the commencement of copulation. It is doubtful if the operation can be speeded up appreciably, and it is most probable, therefore, that it will not be possible to inhibit ovulation in the ferret by post-coitus hypophysectomy. This result, however, implies no criticism of the view that the anterior lobe is directly responsible for ovulation in the ferret as in other animals; the position is the same as in Smith and White's work on the rabbit, *i.e.*, a complicated operation is not sufficiently rapid to remove the pituitary body before it has secreted the relevant substance into the circulation. Ferrets probably fail to ovulate if copulation is interrupted before the end, and it might thus be justifiable to regard the stimulation of the anterior lobe as dating from the end of copulation. In this case the rate of secretion of the ovulation-producing substance would be astonishingly rapid compared with the time taken for its

Table I.—Effect of Hypophysectomy on Ovulation and the Development of the Corpus Luteum.

Number of animal.	Time of hypophysectomy after copulation (hours).	Left ovary.		Right ovary—Autopsy.		Condition of uterus at autopsy.	Condition of pituitary fossa.
		Time removed after copulation (hours).	Ovulation.	Time after copulation (days).	Average diameter corpora lutea (mm.).		
RHF 13	10.15	58	7 r.f.	18	0.77 (5)	No pseudopregnant development	Possible fragment of A.P.
RHF 14	4.00	70	1 r.f.	28	0.78 (6)	"	Devord of A.P.
RHF 15	15.15	58	6 r.f.	21	1.22 (4)	"	Fragment of A.P.
RHF 16	12.00	83	6 r.f.	17	0.86 (4)	"	Devord of A.P.
RHF 18	7.00	78	1 r.f.	11	0.91 (4)	"	"
RHF 19	2.30	116	8 r.f.	8	1.05 (3)	"	Possible fragment of A.P.
RHF 22	1.50	96 (both ovaries)	7 r.f.	—	—	"	Devord of A.P.
RHF 23	1.50	96 (both ovaries)	5 r.f.	—	—	"	"

A.P. = anterior pituitary lobe. r.f. = ruptured follicles.

effect to appear. It is likely, however, that in the ferret stimulation of the anterior lobe by the act of copulation is a much slower process than the trigger mechanism of the rabbit, and we have therefore preferred to adopt the more comprehensive method of dating the experiments from the beginning of copulation. On this reckoning, the ovulation-producing substance is secreted into the circulation in under 2 hours after the first stimulus to the pituitary body. The actual time may be very short. Even on this over-estimate, the time is relatively less than in the rabbit, in which the pituitary body is necessary after copulation for a period equivalent to rather less than 10 per cent. of that taken for ovulation to ensue. The corresponding figure in the ferret is certainly not more than 5 per cent. at the maximum. The significance of this difference is not obvious.

IV. *Effect on the Development of the Corpus Luteum.*

Although segmenting ova were found in the left Fallopian tubes when the left ovaries were removed after ovulation, none of the hypophysectomised animals showed any signs of being pregnant at autopsy. This sterility is probably due to the lack of development of the corpora lutea described below.

According to Hammond and Marshall (1930) the corpus luteum of the pregnant or pseudopregnant ferret reaches its maximum diameter of about 2 mm. in the 3rd to 5th weeks after copulation, fig. 2, Plate 4. Our own experience entirely agrees with this. The diameter of the ruptured follicle 48 hours after coitus is of the order of 1.1–1.2 mm. and the changes in the corpus luteum during its development therefore represent a volume increase from about 0.8 c.mm. to about 4 c.mm. This growth curve is roughly indicated by the upper line on fig. 1.

In the hypophysectomised animals we have obtained a series of corpora lutea up to 4 weeks after copulation, hypophysectomy having been carried out at the times after copulation stated in Table 1. The largest corpora lutea were found in the right ovary of RHF 15 obtained 21 days post-coitum; the average diameter of the four corpora lutea present was 1.22 mm., representing a volume of about 0.95 c.mm. These corpora lutea were thus about one-quarter the normal size. This animal, however, probably had two fragments of anterior lobe left in. The remaining five stages obtained, at 8, 11, 17, 18 and 28 days post-coitum, showed greater degeneration of the corpus luteum, figs. 3 and 4, Plate 4. The least atrophic corpora lutea were obtained from the right ovary of RHF 19 and had a mean diameter of 1.05 mm. (volume 0.616 c.mm.).

The most atrophic were obtained from RHF 13 and 14 (mean volumes 0.239 and 0.247 c.mm.) at 18 and 28 days post-coitum. The mean volumes of the corpora lutea of the hypophysectomised ferrets are plotted in fig. 1 in comparison with the approximate growth curve of the normal. There would appear to be a gradual falling off in size from immediately after ovulation; at the time when pseudopregnancy should be fully developed the corpus luteum is less than one-tenth normal size. Since the corpus luteum fails to develop after ovulation, it is rather remarkable that the atrophic structure persists

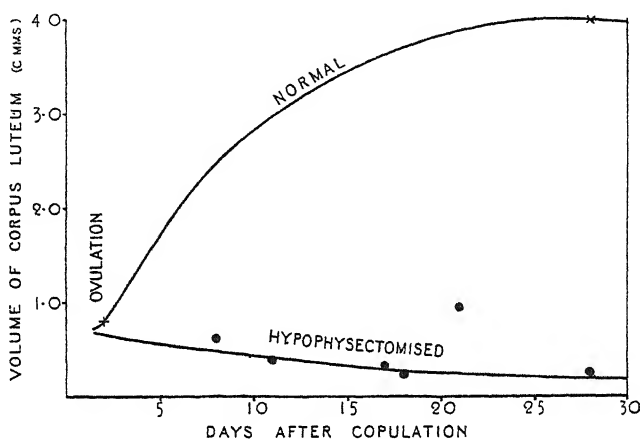


FIG. 1.—Size of the corpus luteum after post-coitus hypophysectomy compared with approximate growth curve of the normal.

for even as long as a month. This persistence of the degenerate corpus luteum after ovulation has, however, been noted by Smith (1930) in the rat, and is probably due to lack of competition from the follicular apparatus. A similar state of affairs was found in the mouse after obliteration of the follicles by X-rays (Brambell, Fielding and Parkes, 1928). We have not yet determined the ultimate fate of the corpus luteum in the hypophysectomised animal.

Histologically, the corpora lutea in the hypophysectomised animal show a curious condition of arrested development. Most of them retain the general structure of the young corpus luteum, *i.e.*, luteal tissue surrounds a central core of fibroblastic tissue, with a general drift of the cells towards this core and the now occluded ovulation point. At 8 days post-coitum the actual cells of the corpus luteum of the hypophysectomised animal are very similar to the cells of the newly ruptured follicle, *i.e.*, little change has occurred in the old granulosa cells. The nuclei are still large and seem in good condition. There is a remarkable lack of cohesion between the cells, a feature which persists in the older corpora lutea. During the next 3 weeks the cells shrink slightly

and the nuclei, in particular, become smaller. At 28 days post-coitum the cells are not more than a quarter the diameter of those of the corresponding normal corpus luteum, fig. 6, Plate 4. The general picture is essentially different from that presented by the corpus luteum degenerating after pseudopregnancy and is typically one of arrested development.

Judging by the lack of pseudopregnancy changes in the uteri of the hypophysectomised animals, fig. 5, Plate 4, the stunted corpora lutea perform no endocrine function.

The microphotographs in this and the preceding papers are the work of Mr. F. J. Pittock.

V. Summary.

(1) Hypophysectomy of the ferret more than 1 hour 50 minutes after the beginning of copulation does not inhibit ovulation, which follows, as usual, in about 36 hours. It would, therefore, appear that the ovulation-producing hormone is secreted into the circulation in less than 2 hours after the beginning of copulation.

(2) This represents about 5 per cent. of the time taken for ovulation to occur after copulation, as compared with a corresponding figure of about 10 per cent. in the rabbit.

(3) Following hypophysectomy, the corpus luteum of the ferret fails to develop, and at 1 month after the operation the volume is less than one-tenth that of the normal corpus luteum of the same age.

(4) The animals fail to get pregnant after hypophysectomy before ovulation, and the uterus shows no signs even of pseudopregnant development.

DESCRIPTION OF PLATE.

FIG. 2.—Corpora lutea of the ferret on the 28th day of pseudopregnancy. $\times 20$.

FIG. 3.—Corpora lutea (median section) of RHF 19, right ovary, 8 days after hypophysectomy carried out 1 hour 50 minutes after copulation. $\times 20$.

FIG. 4.—Corpora lutea (median section) of RHF 14, right ovary, 28 days after hypophysectomy carried out 4 hours after copulation. $\times 20$.

FIG. 5.—Uterus of RHF 14, showing lack of pseudopregnant development. $\times 20$.

FIG. 6.—High power of part of corpus luteum in fig. 4, showing small size of cells and lack of cohesion. $\times 140$.

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Address of the President, Sir Frederick Gowland Hopkins, at the Anniversary Meeting, November 30, 1932.

In accordance with pious custom I will begin my Address by recalling the losses that the Society has sustained during the year behind us. Two of our Foreign Members have passed away and fourteen of our Fellows :—

I will speak first of the former :

KARL RITTER VON GOEBEL. Von Goebel was supreme in the field of plant morphology, and played a leading part in guiding that branch of science on to modern lines. His influence was exerted in bringing the circumscribed and formal studies of morphology into closer contact with experiment and relating them with function. He gave to the subject a philosophical outlook. While a learned student of Form, he was also himself an experimentalist, and one less concerned with the construction of phylogenetic theories than with the study of causation. In illustration his experiments dealing with the effect of environment on symmetry may be quoted. His contributions to science were extensive and various, ranging in their date of publication from 1877 until near his death. His encyclopædic book, “*Organographie der Pflanzen*,” exerted a great influence upon the thought of others. Of von Goebel an informed writer has said that “he leaves behind the memory of a gracious personality to whom the science of botany owes a supreme debt, not only as a great observer, but also as a safe guide to correct channels of thought.”

GRAHAM LUSK. Lusk devoted the whole of his active life, from early manhood to its end, to patient studies of the problems of animal nutrition. As a student he worked successively with Carl Voit and Max Rubner, and in his own work he most profitably combined the methods of both of these investigators ; the chemical studies which derived from the former were checked and extended by the calorimetric technique of the latter. Lusk contributed much of importance to our knowledge of nutrition and metabolism, and especially to that of the intermediary stages in the metabolism of the main foodstuffs. His book, “*The Science of Nutrition*,” exerted a wide influence. He was one of the American representatives on the Inter-Allied Scientific Food Commission in 1917, and his wide knowledge was of great service to that body. Lusk was a man who stood for the highest standards in science, conduct, and all things else, and won the deep respect of all scientific

circles in America. He had many friends in this country, and his friendship was a gift enjoyed by all who possessed it. The circumstances of his election to our Membership were sad. He had received news of it with deep gratitude, and then before many days had passed he was no more.

I will speak of the Fellows in the order of their demise :

ALFRED FERNANDEZ YARROW. A great marine engineer and shipbuilder ; a great benefactor to science, and a great-hearted man. Lady Yarrow in her biography of her husband quotes his first schoolmaster as saying that he "was born with two leading features—a talent for engineering and a thirst for affection—to give and to receive." The talent and the thirst remained with him throughout a long life, the latter being sometimes revealed even to the least intimate of his friends. How early the talent was displayed is made evident in the biography, and it is of interest to recall how as a boy he constructed and installed with the aid of a friend the first private overhead telegraph in London. He was apprenticed to a London shipbuilding firm before he was sixteen, and at the end of that apprenticeship, finding the firm unwilling to help him, and having collected a little capital, he determined to be independent, and, together with a friend called Hadley, started shipbuilding works of his own on the Thames. These works rapidly became a centre of great activity and obtained a reputation which may be measured by the fact that, when Yarrow determined to move to the Clyde in 1905-6, over 400 invitations were received from various local authorities, all anxious that so flourishing an enterprise should come into their midst. It is impossible to give here even a bare list of Yarrow's successes in developing speed and efficiency in ships. His reputation especially grew during the period when he was busy constructing fast torpedo boats and destroyers, but he built many other types of vessel, and many which possessed qualities quite remarkable in their day. In his efforts to increase speed he contrived, and after many years of expensive experiment, constructed the water-tube boiler with which his name will always be associated ; this, however, was but one out of many successes which he fathered.

It was in 1923 that Yarrow handed to the Royal Society £100,000 for the furtherance of research, with the one stipulation that none of it should be employed in expensive building. He was entirely satisfied with the policy which led to the foundation of the Research Professorships carrying his name. Characteristic of him was the modesty which led to his surprised gratification on his election to the Fellowship in 1922. He retained great activity of mind and an adventurous spirit up to his last and ninetieth year.

WILLIAM DAVID DYE, an experimentalist of the very highest rank, began his all too short scientific career as Student Assistant at the National Physical Laboratory. In that home of precision, where the attainment of ultimate accuracy is ever sought, he found an atmosphere entirely suited to his genius. He came under the influence of Mr. Albert Campbell, the beauty of whose experimental work stimulated Dye and started him in the employment of his own talent for the development of new methods. He soon gained a reputation for highly accurate work on the primary electrical standards and units. After the war he succeeded Dr. F. E. Smith as head of the Electrical Standards Division of the Laboratory, and continued to develop methods of great accuracy in such fields as terrestrial magnetism and radio frequency. In everything related to Radio Standards he became a recognised authority. He was elected Fellow of the Society in 1928.

JAMES MERCER. Mercer was a mathematician who did brilliant work while holding a research Fellowship at Trinity College, Cambridge. In subsequent years other claims upon his time, and unsatisfactory health, limited his output. His original work extended and illuminated the Theory of Integral Equations and covered other ground of much importance in pure mathematics. A paper on the Limits of Real Variants which he published in 1906 has inspired quite recent work. Another in 1909 describes work which led to the result known as Mercer's Theorem. He held the post of Assistant Lecturer at Liverpool University and later became Fellow and Lecturer of Christ's College, Cambridge. He was elected to the Society in 1922.

Sir FREDERICK ANDREWES. Andrewes was a pathologist, more especially concerned with bacteriology, who always kept in close touch with the clinician. It was his constant desire that his science should serve the immediate needs and promote the current progress of practical medicine. This did not involve a lack of interest in the wider problems of bacteriology as a branch of biology, but it perhaps led to the circumstance that his scientific work, though always significant, greatly helpful, and covering a wide field, was for the most part concerned with practical details. Of wide interest, however, were studies such as that carried out with T. J. Horder, which provided a basis for the classification of pathogenic streptococci, and the serological analysis of dysentery and the typhoid group of organisms which he pursued during the war. Andrewes was a man of wide culture and a charming companion. He was elected to our Fellowship in 1915.

GEORGE CLARIDGE DRUCE. Although engaged in the exacting business of a retail pharmacist throughout his life, his innate love of Nature in the wild,

displayed from his boyhood onwards, led Druce to become a leading authority on the British flora. Of this he possessed an intimate personal knowledge gained by frequent visits to all parts of the country. His book "The Flora of Buckinghamshire" of which the last edition was published as late as 1930, is a classic of its kind. He wrote much upon other local floras and kept in constant touch with every advance in field botany. His business was in Oxford, and he enjoyed close association with University authorities. In 1895 he was made Curator of the Fielding Herbariums, and was attached to Magdalen College with an honorary M.A. degree. Later he became D.Sc. The life of Druce was of a quality which the Society did well to honour. He was elected to the Fellowship in 1927.

ERNEST HOWARD GRIFFITHS. Griffiths had been for some years a private science tutor at Cambridge before he displayed the urge for research. In 1887 he started work in the Sidney College Laboratory, almost in solitude, and began an attack on the problem which directly, or indirectly, occupied most of his years as an investigator. This was to determine Joule's Constant with the electrical method raised to a high degree of accuracy. The needs of this problem led him to investigate platinum thermometers and the measurement of platinum temperature as introduced by Callendar, and he published several papers on the subject. His publication "On the Value of the Mechanical Equivalent of Heat" which appears in the 'Philosophical Transactions' for 1893, is of permanent importance. In 1901 he became Principal of University College of South Wales, and the duties of that post interrupted his research activities for several years. Later, however, when a research laboratory was built at his University, he did further work, and just before the war published, along with Dr. Ezer Griffiths, papers upon the heat capacity of metals. He passed his later years in retirement at Cambridge, suffering unfortunately from bad health, but retaining all his scientific interests. He became a Fellow in 1895, and was later awarded the Hughes Medal.

SIR HORACE PLUNKETT was one whose qualifications for sharing our Fellowship were other than those most commonly recognised. He had no close knowledge of science or its methods, and perhaps no special interest in it for its own sake. He recognised, however, that science could help towards the accomplishment of the great aim of his life, and realised this perhaps more and more during the progress of his efforts. Moreover he saw, as a sympathetic biographer has remarked, that science applied to things must be controlled by science applied to men. As is well known, the object of his ambition was the regeneration of Irish rural life. To this end he started a great movement

which was to unite all classes, creeds and parties in an endeavour to promote economic and social progress in his native country, and particularly to secure higher standards of living and enterprise among those who cultivated her soil. Under his inspiration the Irish Agricultural Organisation Society which he started in 1894 secured the unity he hoped for, and the result was a new and real co-operation among agriculturists. At a later date the disintegrating forces of politics interfered with his beneficent policy, and the sad aftermath of the war led to the destruction of his home in Dublin. He resumed his efforts, however, when thereafter resident in England, and continued them to the end. Surely, if indirectly, he did much for the advancement of agricultural science, and his services were recognised by his election to the Society in 1902.

SIR WILLIAM WATSON CHEYNE. The science of Pathology towards the end of the last century stood deeply in debt to this distinguished surgeon. While himself a pioneer in bacteriological research he did more perhaps than anyone else during the eighteen seventies and eighties to make known in this country the bacteriological researches which were proceeding so rapidly in Germany and France and to interpret their results. English pathologists enmeshed in the details of morbid anatomy, and more used to descriptive than to experimental methods, were then contributing little or nothing to bacteriology. The influence of Watson Cheyne, however, greatly stimulated interest and research in the subject. In his younger days he wrote and worked under the inspiration of Lord Lister, whom he served as House Surgeon, first at Edinburgh and later at King's College, London. Had he possessed independent means, then very necessary for complete devotion to pathology, there is little doubt that he would have chosen a scientific career; but he was compelled to earn his living as a surgeon, and in that calling he attained to eminence. He was elected to the Fellowship in 1894.

JOHN WALTER GREGORY. It has been justly claimed that Gregory of all British geologists was the most widely known. Urged by a natural lust for travel, but always in pursuit of significant knowledge, he explored the world. That he was a geographer as well as a geologist explained, perhaps, the nature of his special interests; but it must not be forgotten that he was incidentally also a palæontologist, petrologist and mineralogist. He was well equipped, therefore, to appreciate all the evidence concerning earth structure and earth history that travel presents to eyes and mind. He knew Spitzbergen and no less Thibet. The face of nearly every inhabitable country was familiar to him, and he himself was well known at every centre where the subjects of his

interest were being studied. Gregory began his scientific career as an assistant at the British Museum. Later he became Professor of Geology in Melbourne, and finally, for a quarter of a century held the chair of that subject in Glasgow. As a teacher of the subject he was highly successful. All who knew him well testify to his great and accurate erudition, and to that originality of outlook which is manifest in his writing. Some two hundred published papers bear witness to his intellectual fertility. Though 68 years old he was yet on a venturesome quest when an unhappy accident led to his death. He was drowned last June by the overturning of his canoe when on the River Urubamba in Northern Peru. He was elected a Fellow in 1901.

SIR RICHARD THRELFALL. One might be content to describe Threlfall as one of the greatest of electro-chemists ; as one who, combining chemical insight with the aptitudes of an engineer, and much scientific acumen, notably promoted the progress of industrial chemistry. Such a description would, however, be inadequate. In more than common measure personality and character contributed to Threlfall's influence. Contact with him was for his contemporaries a refreshing stimulus, for younger workers it meant sure help and encouragement. While bent on successful accomplishment for himself, he loved success in others.

In his student days at Cambridge, he was an athlete of note. Many will have enjoyed the tale, as told by Sir Joseph Thomson and others, of his successful fight for the recognition of Rugby football as a sport entitled to the award of "Blues."

After acting as Demonstrator in the Cavendish Laboratory he went to Sydney as Professor of Physics. In 1889 he returned to England and joined the firm of Albright and Wilson, the great producers of phosphorus, at Oldbury. Among the most interesting and enterprising of his researches while at Sydney was his comparison of values for gravity at different places by means of a quartz thread balance. He was able to claim high accuracy for these determinations. During his later years he did further work with the same balance. It remained at Sydney after Threlfall resigned his Chair there, until, in 1923, at the suggestion of Sir Frank Smith, it was brought to this country for study. Its subsequent history is told in a fascinating paper by Threlfall and A. J. Dawson in our 'Philosophical Transactions' of the current year. In his earlier years he invented the rocking microtome, an instrument which has been of priceless value to many branches of biological study. He himself recently discussed the history of the instrument in the 'Biological Reviews,' published by the Cambridge Philosophical Society. I have referred to his

dealings with these two very diverse instruments of precision in illustration of the breadth of Threlfall's interests. On his triumphs in industrial chemistry I need not dwell. The debt that we owe to him, however, cannot be made clear without reference to his war work. The nation's needs were greatly served by his experience and his qualities. The Army, Navy and Air Force all alike benefited by the response of his practical genius to the call of some of their most urgent needs. Threlfall in recent years made a point of attending the Society's meetings as often as was possible, and was our frequent and ever welcome companion at Club dinners. He will be greatly missed. He was elected a Fellow in 1899.

JOHN CHARLES FIELDS. Fields was a highly gifted mathematician well known in particular for his famous treatise on the "Theory of the Algebraic Functions of a Complex Variable." He came early in life under the influence of distinguished German teachers and acquired a deep desire to do, and to encourage original and creative work in his subject. This enthusiasm he brought to his Chair in the University of Toronto where he always advocated, and in every way promoted, the claims of research. It was largely due to his influence that the Legislature of the Province came to make annual grants to the University ear-marked for the encouragement of original work, and he initiated a movement in the direction of having research professorships attached to the Royal Canadian Institute similar to those administered by ourselves. In the teaching of mathematics in Canada and the United States his influence helped to bring about successful reforms. He became a Fellow in 1913.

Sir RONALD ROSS. It has been given to few as it was given to Ross to produce in a few patient years a great gift for humanity; a gift at once complete, straightway ready for use, and of incalculable benefit. Ross, like many other discoverers, was fortunate in his times and contacts. Contemporary thought and knowledge had shaped a course for him, and no man's work could owe more to the inspiration of another man than that of Ross to Manson. Nevertheless Manson himself declared that his own chief claim to a share in the discovery of the secrets of malarial transmission was his discovery of Ross. It required indeed the special gifts of Ross, his fine technique, his determination, and his patient and observant eye to establish so conclusively, and in what was relatively so short a time, the evil influence of *Anopheles*. Let us recognise the happiness of the circumstance that brought the older and the younger man together. It is impossible in brief paragraphs to follow the stages of Ross's investigation. It offered many difficulties; there were misleading assumptions

to disprove as well as new facts to discover. In particular there was long delay from the circumstance that the delinquent sought, the true carrier of the organism, was a mosquito of no common species : in India its number amounted to no more than a fraction of 1 per cent. of those which swarmed round the investigator. In little more than two years, however, the work in essentials was complete. It only remained for good administration to make use of the results. Though this has not always been forthcoming, yet much has been done, and the world, and especially the British Empire, has received a priceless favour. Ross became a Fellow of the Society in 1901, was Vice-President 1911-15, and received the Royal Medal in 1909.

ALFRED CHASTON CHAPMAN. Chapman's activities were exceptional in that, although engaged from his student days onwards in a professional practice, which grew to be large and exacting, he made time for personal researches which contributed not a little to the advance of pure science. He was deeply interested in some of the wider issues of both chemistry and biology, and he formed independent views concerning them. At the same time he took pride in his profession, and did all he could to promote within it high standards of education and practice. In Presidential Addresses to the Society of Public Analysts, after stating his belief in the educational value of the subject, he urged that Chairs of Analytical Chemistry, directed to giving sound technical training to future professional chemists, should be established in Universities. His interest in mycology and applied bacteriology and a belief in the practical importance of these led him to advocate with equal conviction the foundation of an Institute of Microbiology, which unfortunately this country still lacks.

Chapman gave his time unsparingly to enterprises in which he believed, and he was a highly useful member of a great number of Boards and Committees. He became President of the Institute of Chemistry, of the Society of Public Analysts, and of the Royal Microscopical Society. His personality, unique in many ways, carried a charm which none could fail to recognise. He was elected in 1920.

SIR DUGALD CLERK was a man of great inventive power, whose researches on internal combustion engines and on every aspect of the use of gaseous fuels have contributed greatly to the progress of industry and to human comfort and enjoyment. His work on the specific heat of gases, and that on explosion pressures, are instances of his important contributions to pure science. In the War he served as Director of Engineering Research at the Admiralty, and was Chairman of the Internal Combustion Engine Committee of the Air

Ministry. He was elected to our Fellowship in 1908, and received a Royal Medal in 1924.

The sum total of accomplishment involved in the work of all these, our comrades who are no more, is surely great and worthy of all remembrance.

Mr. Aldous Huxley, writing in just praise of the literary, as distinct from the scientific, work of his grandfather, remarks that Thomas Henry Huxley *was* a hero of science; but *is*, and will remain, a hero of literature. He develops the theme that individual scientific accomplishment, unlike literary accomplishment, is fated to be forgotten, because it is always but a step in progress, and loses its importance as knowledge grows and widens. The truth of this view is, I think, but limited. Great personal accomplishment in science is not forgotten, even if for obvious reasons it is stored in fewer memories than is great literature. Yet I think the historians of science should meet with all encouragement, and not alone because of their piety towards past labours. The perspective of history is illuminating and a corrective at all times. It is especially valuable in maintaining sound judgments in times of revolution; and science to-day is revolutionary. I hope that you will agree with me that Chairs in the History of Science are among the needs of our time.

Whatever of truth there may be in Mr. Huxley's dictum is illustrated *mutatis mutandis* by the life of Christopher Wren. By the majority of his fellow-countrymen Wren is remembered as the great architect; relatively few have kept in memory his greatness as a pioneer in experimental science. But we of the Royal Society in the year of the tercentenary of his birth should surely have in mind his services to the Society, at its birth, and during its infancy. No one who has read our early history, or who alternatively is familiar with the life story of Wren, can doubt that the measure of those services was truly great. You will remember how that group of enthusiasts who had the advance of experimental science at heart attended together a lecture by Wren at Gresham College, and immediately after it sat down to plan for the future. At this meeting, for which attendance at Wren's lecture had served as a sort of ceremony of dedication, the formation of our Society was, in principle at least, decreed. All concerned doubtless felt the refreshing quality of Wren's optimism. Once the actual work of the Society began, his activity inspired every meeting. His keen but rational delight in experiments of all kinds, his mechanical and artistic skill, his quite extraordinary versatility and, not less important, his lovable nature and unselfish helpfulness: all these qualities he

brought to the service of the infant Society to its immense advantage. Wren's own original work was, it is true, not fated to influence the future of science as did that, say, of his contemporary, Robert Boyle, but his personal qualities were exactly adjusted to the service of experimental science when making its first tentative endeavours.

We have arrived at a period when the tercentenaries of the birth of original Fellows, and of those elected early, have occurred, or will occur, frequently in the course of a small span of years. It is beyond our power and resources to organise a public celebration for each such case, however great the desire to do so. But on right occasions our own memories should wake.

And this year we should have specially in mind one other of our early Fellows, the dates of whose birth and death nearly coincided with those of Wren, but whose whole personality differed widely from his save in the fertile curiosity concerning Nature which they equally shared. There would be, in any case, small need to remind you of Antony van Leeuwenhoek's happy relations with this Society. The need is the less because the pious and prolonged labours of our Fellow, Mr. Clifford Dobell, have given us a book in which we may find the quaint, lovable and altogether remarkable personality of the man made for us almost a living figure. You will recall that Leeuwenhoek in 1673 got into touch with Oldenburg, our active Secretary of those days, and then, for fifty years, constantly transmitted to the Society all his microscopical observations and discoveries. Very numerous papers and letters from him are preserved in our archives. His skill in making lenses, by methods which he kept secret, was almost miraculous. Those who were fortunate enough to attend a recent meeting of the Society when Professor D'Arcy Thompson gave a demonstration, saw proof that one of these simple biconvex lenses could show the structure of a diatom with a definition equal to that given by a modern compound microscope. Leeuwenhoek was elected a Fellow of the Society in 1679.

Allow me yet a reference to one other among the great men whose association with the Society illumined its past. Elected in 1688 was Marcello Malpighi, the manuscripts of whose contributions to our early Transactions are another of the great treasures of our archives. In contrast to Leeuwenhoek, who made and used his microscopes as an amateur, Malpighi held Chairs of Anatomy in several of the great Italian schools of medicine, and was physician to the Pope of his day. His researches covered a wide range in the comparative anatomy of animals and plants, and his richly illustrated accounts of the development of the chicken in the incubated egg, of which the original

manuscripts and drawings are, let me remind you, in our library, made him the founder of the science of embryology. He also first saw the connexion of arteries and veins by the network of capillary vessels, though a clearer account of these and of the blood corpuscles passing through them was given by Leeuwenhoek. The Society may well be proud that these two great pioneers in biology and in the use of the microscope were enrolled among its early Fellows, and that its Transactions gave many of their greatest discoveries to the world.

The Report of Council contains the customary account of the activities of the Society during the year. I will refer here to a few items of special interest.

An important benefaction has increased our opportunities for supporting the work of highly qualified investigators. The late Mr. Gordon Warren, shortly before his death, placed at the disposal of the Society the sum of £1,400 annually for a period of seven years for the purpose of maintaining a Research Professorship or two Research Fellowships. Council gratefully accepted the proposal, and the first two Warren Research Fellows, Dr. A. J. Bradley and Dr. W. Hume-Rothery, were appointed in June.

Mr. Warren's death occurred with tragic suddenness while he was considering further plans for the benefit of scientific research. In his will he left a large sum in the hands of his bankers, which, subject to a life interest, will ultimately be devoted to the purpose of science. It was his expressed wish that the Royal Society should be consulted in the matter of its disposal, because of his conviction, which most will feel to be justified, that the Society is especially well qualified to advise on such matters.

The award of a Messel Research Fellowship to Mr. C. N. Hinshelwood, who was elected to the Fellowship of the Society three years ago, will, I believe, give general satisfaction. The Society is fortunate when it is able by such appointments to secure for distinguished investigators increased opportunities for research.

Various grants from our Trust Funds are enumerated in Council's Report. I may mention that the Royal Society Mond Laboratory at Cambridge, towards the building of which we provided £15,000 from the Mond Fund, is now practically complete and will be opened early in February next.

As the result of the Government's Conversion Scheme, and for other reasons, important changes have been made in our holdings of Stocks. The result has been a slight reduction in the income of some of our Trust Funds, but Council are convinced that the changes will be to the advantage of the future financial position of the Society.

I would draw attention, as important to all Fellows, to the Report of the Library Committee which has been adopted by Council. In particular I will emphasize the desire of the Committee that the Library should possess a copy of every book of importance written by a Fellow of the Society. It is hoped that authors will look upon the presentation of such books as a normal obligation.

An event of the year with which the Society is deeply concerned is the retirement of Sir Richard Glazebrook from the Chair of the Executive Committee of the National Physical Laboratory. Sir Richard's services in that capacity, continuing those he rendered as first Director of the laboratory, are of national importance, and their value cannot be overestimated. For the labours which have contributed so much to the creation and development of one of this country's most valuable assets the Royal Society, because of its responsibilities, must ever be especially grateful. The gratitude, however, like the services, should be national.

In now attempting a brief review of some few aspects of scientific progress, I should like to refer, for my text as it were, to the two organised discussions which were held during the current year. The success of these has further justified, I feel, the policy which decided that such discussions should be organised from time to time. In particular they are valuable when they tempt distinguished workers from abroad to visit the Society as contributors to debate. It might be well, I think, if we agreed to break with tradition and on these occasions extend the hours of meeting. I should have found it impossible, in any case, to omit some reference to the discussion, opened by Lord Rutherford in person, on the structure of atomic nuclei. The occasion was remarkably timely, for after a date was fixed for it, but before that date arrived, certain pregnant researches had brought forth supremely important data, with a final rapidity which I think had been unexpected by all concerned. The revelation of these new experimental results and of their great significance gave a dramatic character to the discussion which was felt by all who were present. The atomic nucleus for a long time had seemed to be an impregnable fortress; but missiles of high destructive power have been gradually contrived by almost magical skill in the army of attack, and the fortress, in spite of its formidable potential barrier, is crumbling. It is interesting for the spectator to realise how much is learnt by the commanders of the attack from the nature of the missiles (parts of itself) with which the fortress replies to the bombardment. Even were it within my ability this is, of course,

not the place for an attempt to summarize the discussion. One cannot help recalling, however, the sense of progressive accomplishment which was conveyed in Lord Rutherford's opening address, as for instance when he dealt with the nuclear origin of the γ -rays. Nor can one forget moments of actual excitement as when, recalling a twelve years old prophecy of his own respecting the probable existence of neutrons, he referred to Dr. Chadwick's recent success in producing these entities (of which the mass is unity and the charge zero) by bombarding beryllium with α -particles from polonium. During the discussion a full account of this success was heard for the first time. Exciting again was the moment when your ex-President at the close of his remarks referred to the striking results obtained by J. D. Cockcroft and E. T. S. Walton, also in the Cavendish Laboratory, and but a very short time before the meeting. These investigators (as is by now well known) having constructed an apparatus capable of providing a steady stream of protons, of energy up to 600 thousand volts, successfully employed the stream in the disintegration of the lithium nucleus. "The simplest assumption to make (I quote Lord Rutherford) being that the lithium nucleus of mass 7 captures a proton, and the resulting nucleus of mass 8 breaks up into two α -particles. On this view the energy emitted corresponds to about 16 million electron volts . . ."

It is not unjustifiable to say that before the moment of Cockcroft and Walton's success, man did not know how to release atomic energy on his own initiative, whereas now, though doubtless in a limited sense, he possesses that power. At the same time the phenomenon of transmutation seems to be at hand in full reality. The occasion of this discussion cannot fail to stand out as of much significance in the annals of the Royal Society and in the history of this country's contributions to science. Hence I think my reference to it is fully justified.

The second discussion dealt with the growth of knowledge at a different level of present accomplishment; but with phenomena that are highly significant. It was concerned with recent studies of the nature and properties of those highly active catalysts—the enzymes—the presence of which in each living unit converts a system, which without them would be static, into an organism which is so characteristically dynamic. Anyone who reads in succession the records of these two discussions as found in our 'Proceedings,' will perhaps be tempted to wonder how soon, if ever, intellectual concepts, based upon the phenomena which were the subject of the first, are fated to invade, and perhaps revolutionize thought in the great field of which the second covered part. Will the data of atomic physics ultimately illuminate the processes of life? An interesting question for all biologists. At present we know nothing to

suggest a certain answer. I have indeed met not a few who had a strong *a priori* conviction that life, in some way, in some limited sense at least, makes use of atomic energy ; that such ability might indeed be the special stamp of life. Some twelve years ago a distinguished Dutch physiologist, the late Professor Zwaardemaker, thought he had proved that the weak radioactivity of potassium is an indispensable stimulus to certain vital activities ; but the importance of this influence would seem to be at most very small. Even Zwaardemaker did not hold that it conditioned life. Its radioactivity is certainly not the main reason for the indispensability of potassium in living systems.

Although they bear very remotely, if at all, on the question I just now raised, it may be logical here to mention certain recent experimental studies which seem to have proved that living tissues may be the seat of radiations able to produce effects at a distance, and to suggest that certain activities in one cell of a tissue can thus influence activities in neighbouring cells. It was claimed some time ago by Gurvitsch, a Russian biologist, that when growing cells divide they emit rays which accelerate the processes of division in other cells. The existence of these mitogenetic rays, so called because of the claim mentioned, met at first with general disbelief, and a year ago I might have been disinclined to mention the subject ; but work by many during the last year seems to have brought satisfactory proof that chemical reactions in living tissues are indeed accompanied by radiations, and that events in one cell may thus influence other cells without material transmission. The phenomena as described are doubtless related to that of chemiluminescence, which many non-biological reactions display, and may perhaps have affinities with the emission of more intensive radiations by specialised cells in the luminiferous organs of animals or by luminiferous bacteria. The much more general invisible radiations under reference have been now studied by physical methods. Their emission from active cells has been (it is claimed) demonstrated by means of Geiger's Counter ; their wave-length measured, and by methods which I must not stop to describe, their specific spectra in various cases duly mapped. It has even been claimed, for instance, that a characteristic spectrum of a radiation from a tetanised muscle is identical with that yielded *in vitro* by a reaction (the breakdown of creatin phosphate) known to occur in active muscle. Many published statements of this kind must be received with hesitation until fully confirmed ; but that activities in living cells may be accompanied by radiations recognisable by physical means is now, I think, a fact which is proved. This alone will certainly lead to many fresh lines of enquiry. It is

not yet proved, however, that the phenomena as described, are of fundamental importance, nor even that they are associated with all forms of life.

What, on the other hand, we do know for certain, is that in all living systems in which dynamic events have been adequately studied, the influence of colloidal catalysts is found to be dominant. These catalysts ("enzymes" if you will) exert a specific control over complex chemical reactions, of which the exact co-ordination in time and space is one of the primary characteristics of an organism. It is, I think, difficult to exaggerate the importance to biology, and I venture to say to chemistry no less, of extended studies of enzymes and their action. Of the chemical reactions displayed in an organism few, if any, proceed uncatalysed, while they are reactions so completely and harmoniously organised that all are maintained in complex dynamic equilibrium. If chemical thought is to function with effect in helping towards a description of living systems, it must dwell especially on this chemical co-ordination which, like other aspects of organisation, illustrates that subservience of parts to the whole which characterises an organism. The organising potentialities inherent in highly specific catalysis have not, I believe, been adequately appraised in chemical thought. The concentration of a catalyst or, alternatively, the extent of its active surface will determine the velocity of changes due to its influence, but highly specific catalysts determine in addition just what particular materials, rather than any others, shall undergo change. In this respect they are like the living cell itself, for they select from their environment. Finally the specific catalyst, in virtue of its own intimate structure, determines which among possible paths the course of change shall follow. It has directive powers. Even in a cell juice, or in an extract from living tissues from which all cell structure is absent, experiment has shown that a group of contributory reactions, including syntheses, may proceed in due and just sequence and so lead to the same end result as is normally reached in the intact living system. A striking degree of organisation may indeed be attained in such preparations under the directive influence of the more soluble enzymes derived from the cell or tissue. Much more than must a structured colloidal system, like the intact cell, in which a number of catalysts with such controlling powers are present in circumstances exactly adjusted to a final result, be one in which reactions are conditioned and organised to a high degree without the aid of unknown, or any other influences. I do not expect that all will feel able to admit as much as I myself would like to claim, namely, that the control of events by intracellular enzymes, exerted in the specialised colloidal apparatus of the cell by itself secures the status of the cell as a system which can maintain itself

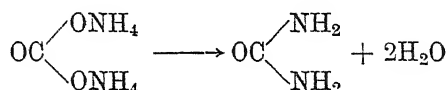
in dynamic equilibrium with its environment. I am not denying for a moment that the cell has esoteric qualities which may call for organising influences of a greatly different kind, exerted maybe at some higher level. It is at any rate sure that the inter-related activity of highly specific catalysts represents a notable device of Nature which has supported during the course of evolution those dynamic manifestations which characterise living things.

The discussion on enzymes greatly profited from the presence of Professor Willstätter, who, together with members of his school, has done so much to advance our knowledge of these agencies. I have sometimes heard it suggested that the advance in question, from a chemical standpoint at any rate, represents a relative failure, apparently because no enzyme has yet been isolated in a state to conform with the classical criteria of "purity." If this be the reason for any suggestion of failure, there is surely some misunderstanding. Isolation, individualisation and purity are words which, if used at all in this domain, may well need to be given meanings differing not a little from those which are applicable in classical organic chemistry. Few will doubt to-day that the specific influence of a catalyst is due to its specific structure. All indications, however, point to the circumstance that the active structure of an enzyme is supported by a colloidal "carrier" which stabilizes it. It is indeed likely that in very many cases, if not in all, the active catalytic mechanism is a specific configuration at part of the surface of a colloidal particle, or, alternatively, part of a structural surface in the histological sense. If so we should no more expect to isolate them in a pure state than so to isolate the active areas on a catalytic metallic surface. It is true that enzymic activity may be displayed by agencies which are not all strictly of one type. It is not unlikely that in certain cases the specifically active groups may be inherent in the structure of a complex but relatively stable molecule, such as that of an exceptional protein. Cases are known indeed in which a protein many times recrystallised retains specific enzymic activity. As was pointed out in the discussion, however, in one such case at least it has been shown that the protein structure can be to a large extent destroyed without disappearance of the activity. Crystallisation in such a case does not yield an entity which would reveal its active structure to the ordinary methods of organic chemistry. What is essential for enzyme studies at their present stage is an assurance that a single entity alone is responsible for this or that observed activity. To this end the technique developed by the school of Willstätter has greatly helped. While we are waiting for the knowledge which may ultimately yield, on lines acceptable to current chemical thought, a method for characterising these exceptional

entities as units, the actual configuration which confers activity on this or that enzyme can be, and in many cases no doubt soon will be, determined by indirect methods. The future of such methods was at least foreshadowed during the discussion, for instance in the contributions of Professor Waldschmidt-Leitz, and Dr. J. H. Quastel.

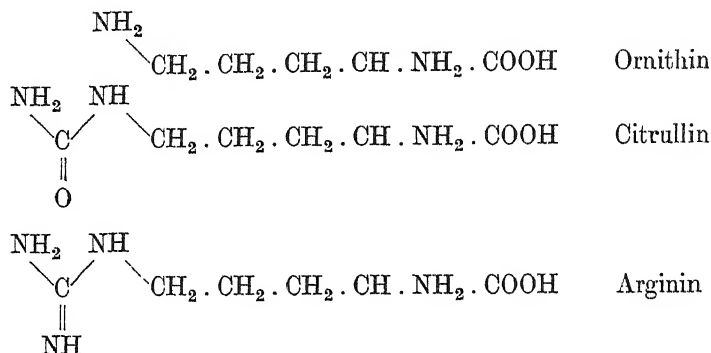
I would like now to illustrate a little further the nature of current progress in animal biochemistry by a reference to investigations dealing with related, but somewhat different aspects of the control of dynamic phenomena in living tissues.

From the researches published during the year I might select many to show that efforts to disentangle the complexities of these phenomena can in their own way be as profitable as any branch of chemical endeavour. I think it will be more useful, however, if you will allow me to refer more particularly to one research which is typical of many in respect of its methods and its success. In this the investigator approached on new lines a fundamental problem which for the last sixty years has been the subject of speculation, and no less of experiments which up to a point were informative. The problem was to discover the nature of the final chemical steps which lead to the production of urea in the animal body. That the mammalian liver can convert ammonium carbonate into urea has been many times experimentally proved, and it is equally sure that ammonia and, of course, carbon dioxide are continuously produced in metabolism. Therefore most of us have long been content to believe that urea arises by the direct removal of the elements of water from the molecule of ammonium carbonate on the lines of the simplest of reactions :



That urea does indeed arise in the liver by a synthesis from ammonia and carbon dioxide remains certain ; but the research under reference, brilliantly carried out by Krebs of Freiburg-im-Breisgau, has shown that its production is on no such simple lines as those mentioned. It calls for a mechanism involving a most interesting interplay among activated molecules. The facts as revealed have just that degree of unexpectedness—if I may use the phrase—which was to be expected in a biochemical phenomenon. I often find myself compelled to assert that though biochemical events are, of course, limited by chemical possibilities, they are not safely to be predicted by chemical probabilities, even when these are strong. That is why experimental biochemistry must remain an independent scientific discipline.

In order to make clear to you the essential results of Krebs' research, omitting of necessity most details, I will ask you to consider the molecular structure of three biological substances : Ornithin, Citrullin and Arginin :—



Note first the structure of ornithin : $\alpha - \delta$ — amino valeric acid. In the presence of ammonia and carbon dioxide, and when activated by agencies in the hepatic tissue, ornithin is converted into citrullin, which, as a ureido-acid, already carries the carbamide structure. Urea does not arise directly from this, however ; another stage intrudes.

Citrullin takes up another molecule of ammonia (with elimination of water as at the first stage) and the structure of arginin with its guanidin grouping is thus established. Now arginin is the normal substrate for the well-studied and very active hepatic enzyme, arginase ; and under its influence the guanidin group is hydrolised. Urea thereupon splits off from the arginin molecule and ornithin is reproduced. The sequence is then re-established. Urea is thus produced continuously from the ammonia which arises in the deamination of the amino acids of protein, and from the carbon dioxide of metabolic oxidations in general, but on lines which may seem strangely complex. It would be too much to say at present that this is the only line of origin for urea in the body, but we know now that it is the main line. In maintaining the sequence of reactions, ornithin can function in minute amounts ; acting therefore essentially as a catalyst. The nature of the relations involved in this mechanism is characteristic of the living cell. Analogies may be found, for instance, in those fundamental oxidation-reduction systems which were mentioned in my Address last year.

In another respect the example I am putting before you illustrates the nature of current biochemical studies. The data were obtained by the methods of micro-analysis and only a few milligrammes of hepatic tissue were employed in

individual experiments. Yet the results were consistent and reproducible and experimental errors well under control. The high accuracy to be obtained in ordinary organic analysis by micro methods is now well recognised, but it is becoming clear that technique is so developing that kinetic studies can be made equally accurate on a similar scale. To studies of living systems this offers advantages which cannot be overestimated. One further point: it is becoming more and more a matter for confidence that when tissues with cells intact are quickly removed from the animal after its death and placed straightway in a fluid medium of carefully proved adequacy it only remains to provide an adequate supply of oxygen which shall reach each unit of the tissue, to secure the continuance of the events which had been proceeding *in vivo*. Indeed we are gaining sufficient knowledge of the requirements of such excised tissues to justify the claim that the course of metabolism observed in them during extensive periods of survival need differ in no way from the normal. All such requirements so far as they are known, were provided in the typical research to which your attention has been turned.

I would point out then that we can proceed from the study of tissue extracts in which it is easy to deal with the kinetics of isolated reactions, each determined by its appropriate catalyst, to studies of other tissue extracts, made with discrimination, in which the progress of a variety of reactions retains not a little of the organisation which characterised them during life, and thence to other studies in which we follow the kinetics of reactions controlled by the intact and still living tissues or cells. Thus and otherwise has biochemistry escaped from the dilemma voiced in earlier dogma, namely that since chemical methods must at the very moment of their application convert the living into the dead, they can do nothing to elucidate the dynamic events of life. The escape is more real than may seem on a superficial view, and especially real perhaps to those who are themselves applying modern chemical methods in the biological field.

Statement of Award of Medals, 1932.

The Copley Medal is awarded to Dr. George Ellery Hale, For. Mem. R.S.

Dr. Hale's first notable achievement was in 1892, when he brought the spectroheliograph to success. This instrument gives a picture of the sun by the light of one spectrum line, and allows the bright clouds of hydrogen and calcium in the upper regions of the sun's atmosphere to be photographed as

projected on the disc. The idea had been suggested and tried much earlier, but Hale was the first to make a workable automatic instrument of this kind. [H. Deslandres was also working with great success on similar lines. Nothing would be gained by a minute analysis of questions of priority, which are not of primary importance. We may compare the case with that of Darwin and Wallace, or with that of Janssen and Lockyer.]

About the year 1895 Hale organised the building of the Yerkes Observatory and of the great refracting telescope there, to which an improved spectroheliograph was adapted. To this period belongs also a masterly investigation of the spectra of certain faint red stars.

The organisation of the Yerkes Observatory would have exhausted the activities of many men. In Dr. Hale's case it was only the precursor of a much larger enterprise, the Mt. Wilson Observatory, with many unique instruments, such as the 150-foot tower telescope and the 100-inch diameter reflector.

At the Mt. Wilson Observatory Dr. Hale made his great discovery of the Zeeman effect in sunspots by observing the circular polarisation of the edges of the broadened spectrum lines, where they cross the spot. Regions of thousands of miles across were thus shown to be the seat of intense magnetic forces, comparable in strength with those used in the dynamo machine. This discovery had been developed in many important directions.

In recent years Dr. Hale has developed the spectroheliroscope, an instrument depending on the persistence of vision which allows us to observe transient phenomena scarcely accessible to the spectroheliograph. We may confidently expect that it will contribute to clearing up the mysterious relations between terrestrial magnetism and solar phenomena.

The Rumford Medal is awarded to Professor Fritz Haber.

For nearly forty years Fritz Haber has been renowned the world over as a leader in the field of physical chemistry. Alike at Karlsruhe, where he went in 1894, and at Dahlen from 1911 to the present time, he has been famed as an ideal Director of Research, inspiring schools of great and highly productive activity. His own early studies of the oxidation and reduction of organic substances by electrochemical methods, and the numerous electrochemical studies which followed this important work; such as his researches on gas cells, on the rate of ionic reactions, on the electrolysis of solid salts, on the velocity of reaction at electrodes, and on the use of the glass electrode, have enormously advanced progress in this branch of science.

His profound study of the thermodynamics of gas reactions culminated in his brilliant researches on the synthetic production of ammonia. With van Oort, Haber had commenced to carry out a preliminary investigation on the ammonia equilibrium, but owing partly to discrepancy with figures obtained by application of the Nernst Theorem, further experiments were made with le Rossignol in 1906. In 1908 satisfactory catalysts had been found and the synthesis of ammonia achieved. The far-reaching technical results of these careful thermodynamical studies are in themselves a monument to Fritz Haber; one of the German factories alone can produce more than 1,000 tons of ammonia daily. The influence of this on the food supply of the world is of the highest importance.

Haber's wide interest, combined with his insight and grasp, made possible application of modern physical principles to a wide range of problems of physical chemistry, such as the determination of molecular structure and calculation of lattice energies, the nature of the amorphous state, chemiluminescence, reaction kinetics and electron emission during chemical reaction. During the past few years Haber has been successfully making manifest the rôle of the hydrogen atom in combustion processes.

By the application of thermodynamical principles in the realm of chemistry Haber has thus not only enriched the mass of human knowledge, but also added to the general welfare of mankind.

A Royal Medal is awarded to Professor Robert Robinson, F.R.S.

Professor Robert Robinson has won world-wide distinction by his work in many branches of organic chemistry, particularly by his elucidation of the structure of plant products and of their phytochemical synthesis. His experimental work covers a wide field of endeavour and is especially noteworthy for its sustained successes. No living organic chemist has displayed a greater versatility of thought and of method. His more recent researches on the distribution, the constitution and the laboratory synthesis of the anthocyanins, the pigments of flowers, fruits and berries have excited the keenest interest of chemists and biologists. His work on the structure of alkaloids and the syntheses to which it has led him are classical in character. The synthesis of tropinone has been referred to as the most elegant synthesis in chemical literature. On the mechanism of chemical reaction he has contributed theoretical ideas which, of interest both to chemists and physicists, have opened new avenues of investigation.

A Royal Medal is awarded to Professor Edward Mellanby, F.R.S.

Professor Edward Mellanby's chief claim is based on his proof that the central factor in the development of rickets is a defective diet. He introduced experimental methods, produced rickets by feeding animals on a deficient diet, and showed that the missing factor was of the nature of a fat-soluble vitamin. Previously only clinical observations had been recorded, on the effect of sunlight, and on other supposed factors; there was no sound evidence before his researches that a material substance regulates the calcification of bone. It was Mellanby's fundamental work which during the last decade made possible numerous and important researches by others, culminating a year ago in the recognition of the material substance (Vitamin D) as an isomeride of ergosterol.

A further claim to the award may be based on Mellanby's later researches, which suggest hitherto unsuspected problems, though their very novelty has so far precluded the clear definition and finality which is now the outcome of his earlier works. Thus he has shown the adverse effect, under certain circumstances, of an excessive amount of cereal germs. In the absence of vitamin A the latter, and particularly ergot of rye, produce a degeneration of the spinal cord. Incidentally, this observation provides a satisfactory explanation of the peculiar and hitherto obscure incidence of convulsive ergotism in man. Mellanby has thus indicated the presence, in one of the chief articles of diet, of a substance of general and unsuspected importance. Because of its fundamental nature Mellanby's experimental work may well rank with the best descriptive work in the biological sciences.

The Davy Medal is awarded to Professor Richard Willstätter, For. Mem. R.S.

Richard Willstätter is recognised by all as among the greatest of organic chemists. In a period extending over a little less than forty years he has given ever clearer proofs of his experimental genius.

His earlier studies gave us our present complete knowledge of the molecular structure of atropine and cocaine, and his analytic and synthetic studies of these alkaloids have had important sequels in systematic organic chemistry and in pharmacology. He then proceeded to a series of ingenious researches bearing on the problem of quinonoid character and on the benzene theory and these led in succeeding years to further work on cyclic compounds of much general interest. He early showed himself to be a master of method in organic chemistry.

Probably Willstätter's name will, in the future, bring most readily to mind

the discovery of magnesium in chlorophyll, and this, along with the painstaking and monumental investigations of the structure of chlorophyll and the blood pigment, represents perhaps the high-water mark of his achievement. Coupled with this work was a series of valuable contributions to the study of carbon assimilation. Equally novel and brilliant were his researches on the anthocyanin pigments of flowers and blossoms; a whole new chapter of organic chemistry was written.

Finally, the studies on the enzymes have added greatly to our positive knowledge, enabled us fully to estimate the difficulty of the task, and laid down the lines on which future work must proceed.

It is impossible in a few words to discuss or adequately appraise Willstätter's outstanding services to science; nothing has been said of lignin, the polysaccharides, or the carotinoids and many other fields in which he laboured with great success. It is clear, however, that he has always attacked the more difficult and fundamental problems relating to the intricacies of complex natural products. Although all his work bears on biochemistry, his methods and outlook were those of the organic chemist.

The Darwin Medal is awarded to Dr. Carl Erich Correns.

Dr. Correns was one of the three botanists (the other two being Tschermak and de Vries) who in 1900 independently brought to the notice of biologists the fundamental work of Mendel, which had remained neglected since 1865. From 1900 till the present time Correns has been actively engaged in developing the science of genetics. Some of his more fundamental discoveries are here mentioned.

In 1902 he was the first to elucidate the remarkable phenomenon of the production of red flowers in the first cross between two white-flowered races of *Mirabilis*. He was also the first to show in the crossing of two species of *Mirabilis* that if very numerous genetic factors relating to small morphological differences are present it is impossible to establish segregation in the F_2 generation, unless very large numbers are available. This explains the appearance of supposed "constant" hybrids, as has since been shown by other observers in numerous instances.

Correns was also the first experimenter clearly to establish inheritance which did not follow Mendelian rules. Thus he showed in *Mirabilis* and other plants that variegation of the leaves depending on the failure to develop chlorophyll, is inherited only through the mother because the plastids which carry the chlorophyll are present in the egg cell and not in the sperm. Again,

he demonstrated that paternal characters shown by extra-embryonal parts of fruits produced by crossing (so-called "xenia") were always limited to the endosperm, *i.e.*, to the food tissue formed by the fusion of a second sperm with nuclei belonging to the maternal parent.

But his most important work is probably the elucidation of the inheritance of sex. By crossing a monœcious with a dioecious species of *Bryonia* he showed in 1907 that the females were all homozygous and the males heterozygous for the sex factor. The generalisation that one sex is always homozygous and the other heterozygous corresponds with the normal approximately equal distribution of the sexes in the offspring of unisexual individuals and with the differences between the chromosomes of the sex-cells, and is now well-established doctrine. Deviation from the equal distribution of the sexes Correns showed to be due in *Melandrium* to the more rapid action of the male-determining sperms, and this is a principle of wide application. Again, he was the first to explain the differential fertility of a generation of plants with their parents and with one another by the assumption of two distinct and inherited inhibiting substances in the stigmata of the flowers.

From 1900, when he helped to found the science of genetics on the basis of Mendel's work, for more than thirty years Correns' work, by its sureness in the perception of fundamental problems and by the excellence of its execution, has been that of a master, and several of the key discoveries in the subject are due to him.

The Buchanan Medal is awarded to Professor Thorvald Madsen.

Dr. Madsen has given distinguished service in advancing the Science and Practice of Hygiene for many years up to the present time. His best known scientific work has been on the toxins and anti-toxins of diphtheria and tetanus bacilli and on other animal, vegetable and bacterial toxins and antigens and their antibodies. He initiated and published with Arrhenius, classical work on the theory of toxin and anti-toxin combination, showing that the process resembled the combination of a weak acid and base rather than the union of a strong acid and base, as had been held by Ehrlich.

Madsen was largely concerned with the origin of the Commission on Hygiene, which he directed in Eastern Europe during the latter part of the War.

Since then he has been President of the Health Committee of the League of Nations and President of the Permanent International Committee on Biological Standards, which was in great part due to his initiative, and has served to promote united action in this sphere by the chief countries of Europe.

The Hughes Medal is awarded to Dr. James Chadwick, F.R.S.

Dr. Chadwick is distinguished for his contributions to Radio-activity and Nuclear Physics. Amongst a number of other investigations on α , β and γ rays he was the first to show explicitly about 1920 that the charge on the nucleus was equal to the atomic number, by a quantitative study of the large angle scattering of α -particles by selected elements (Cu, Ag, Au), thus verifying by direct experiment the correctness of Moseley's deduction. He was associated with Rutherford, 1922-1930, in a long series of investigations (1) on the anomalous scattering of α -particles by light elements, which threw the first light on the size and structure of the nucleus, and (2) on the artificial transmutation of the elements by α -ray bombardment. These experiments showed that at least twelve of the lighter elements were transmuted with the ejection of a proton, and laid the foundations of a study which has recently so rapidly accelerated.

In 1928 efforts were started to improve the technique of these experiments by using automatic electrical counting, and methods were perfected by the end of 1930. Dr. Chadwick took an active part in this work and applied the new methods to a more detailed study of the groups of disintegration protons, especially from boron and aluminium, for which he established clearly for the first time the existence of definite nuclear α -particle and proton levels. Finally, this year, when the observations by M. and Mme. Curie-Joliot had indicated certain curiosities, produced by the supposed γ -radiation from beryllium bombarded by α -particles, Dr. Chadwick immediately recognized that the effects observed could only be adequately explained by the assumption that the radiation from beryllium was of a new type—the ejection of a neutron; by a brilliant series of experiments he confirmed this conjecture, and with the collaboration of Dee and Feather was able to establish its essential properties.

The experiments of Dr. Chadwick are characterised by scrupulous accuracy of measurement and interpreted with great care and critical judgment. They form a striking contribution to science. He has also in virtue of his position played a great part in directing and supervising a large number of other important researches in the same field.

The Mechanism of Adaptation to Varying Salinity in the Common Eel and the General Problem of Osmotic Regulation in Fishes.

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Introduction.

The teleost fishes live in two different environments, the fresh water of lakes and rivers, and the salt water of the sea. With few and unimportant exceptions, these habitats are, respectively, very much hypotonic and very much hypertonic to the fish blood. In both cases continual performance of osmotic work is required for the maintenance of the normal blood concentration. The vast majority of fishes are able to exist only in one type of environment, although some variation in that environment is tolerated. The most pronounced exceptions are various species of the eel, *Anguilla*; other species showing similar but generally less ability include the salmon, the stickleback (genus *Gasterosteus*), and some of the Cyprinodontidæ, notably species of *Fundulus*.

All species of teleosts, apparently, show adaptive responses in blood concentration to slight changes in the salinity of the environment, and the work of many investigators, particularly Duval (1925) and Smith (1930), indicates that the adaptive responses and mechanisms in the eel differ only in degree from those in the osmotically less resistant fishes. The "chloride cells" of the eel gills discovered recently (Keys and Willmer, 1932), were found in all species of fish examined, and were present merely in greatest numbers in the eel.

The present paper describes experimental results with intact eels (*Anguilla vulgaris*) in fresh (Copenhagen tap) water and in sea water of normal composition. No concentrations intermediate between the sea water and tap water were used. While the experimental results are confined to osmotic regulation in the eel, it is believed that they are pertinent to the question of osmotic regulation in the other teleost fishes.

Methods.

The eels used were all healthy specimens caught by net. They were kept in stock and during the experiments in large aquarium tanks supplied with filtered running sea or fresh water. Throughout the period of experiments the water temperature varied only between 9° and 13° C. The animals were weighed in water with the minimum of handling.*

Blood samples were taken from the heart by a syringe, coagulation being prevented by a trace of heparin. The serum was separated off at once by centrifuging. Serum refractive indices were measured in duplicate with a Zeiss Pulfrich refractometer at 17·5° C., after calibration with water and with NaCl solution. Serum chloride was determined by the Rehberg (1926) micro-method, using standard NaCl solutions for controls. Freezing point determinations were made with a cryoscope using about 0·6 c.c. of serum; they proved to be accurate within 0·01° C.

"Amytal eels" were narcotised by means of intra-peritoneal injection of freshly prepared sodium amytal following the technique of Keys and Wells (1930). Blockage of the digestive tract was effectively produced without operative intervention by means of a small cigar-shaped balloon filled with air or glycerine. The balloon was placed in the oesophagus and was held in place by a celluloid tubing stalk, short enough to be entirely enclosed within the mouth, tied to the inside of the upper lip by a single stitch.

Experimental Results.

The experiments fall into two groups, the weight-change experiments and the experiments involving measurements of blood concentration. The weight-change experiments will be considered first.

The first experiments were designed to provide answers to the questions:
(1) what is the normal rate of metabolic loss of weight in the starving eel?

* In view of the difficulty experienced by various workers in getting repeatable weights (see, for example, Gueylard, 1924, p. 49), the procedure may be described. The eel was caught in a dip net, avoiding all sudden moves, the net drained briefly on a table near a tared 3-litre Erlenmeyer flask containing about half a litre of water. With wet hands, the eel was then seized through the net, its head brought to the mouth of the flask, the animal slipped through one wet hand (which removes excess water but does not injure the mucous coat) into the flask and the flask stoppered after the mouth and neck had been wiped dry. Weights were recorded to 0·1 gm., but the repeatable accuracy was only of the order of 0·5 gm.

(2) what part does the swallowing of water play in the maintenance of weight (water balance) in the acclimatised eel in sea water and in fresh water?

Observations continued over many days on a number of starving eels showed that the metabolic weight loss is relatively constant and is of the same order in both sea water and in fresh water. At temperatures between 9° and 13° C. the rate of loss was between 0.2 per cent. and 0.5 per cent. of the total weight per day. A typical series of weights is given in the upper line (open circles) in fig. 1.

The lower line (solid circles) in fig. 1 provides part of the answer to the question about the role of swallowing. The eel, previously long adapted to

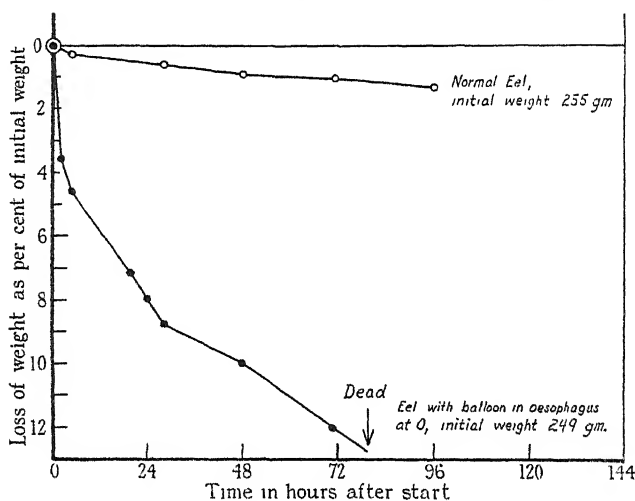


FIG. 1.—Normal metabolic weight loss in the starving eel (open circles). Effect of blockage of the digestive tract on the water balance in the eel in sea water (solid circles).

sea water, had its oesophagus blocked by a balloon at zero time and was returned to sea water. Entirely similar results were obtained with two repetitions of this experiment. It is of interest that death occurred in each case at about the same level of weight loss; the total weight loss at the last weighing before death was 11 per cent., 12 per cent., and 14 per cent. in the three cases. Two of the eels died on the third day and the other eel on the fourth day of the experiment.

Similar balloon experiments were performed on eels in fresh water; they survived in apparent health for a week, at which time the experiments were discontinued. The weight losses were slightly greater than normal, averaging 0.7 per cent. of the initial weight per day, but it was clear that water swallowing is unimportant in fresh water.

These results are in agreement with Smith's (1930) experiments in which swallowing was estimated by the appearance of phenol red in the intestinal contents following the addition of the dye to the external medium. It may be assumed that the weight loss observed in the eels with blocked digestive tracts in sea water is almost entirely due to urinary and extra-renal water loss which normally would be balanced by an equivalent amount of absorption of ingested water. The present experiments indicate a normal water absorption from the gut of about 30 c.c. per kg. per day, a figure similar to but slightly smaller than that calculated by Smith. It must be expected that the loss of water by the balloon eels will tend to decrease as the animal becomes more and more dehydrated, so it is probable that Smith's figure, about 40 c.c. per kg. per day, is more nearly correct.

The experiments relating to water movement in the course of adaptation to changed salinity may now be considered.

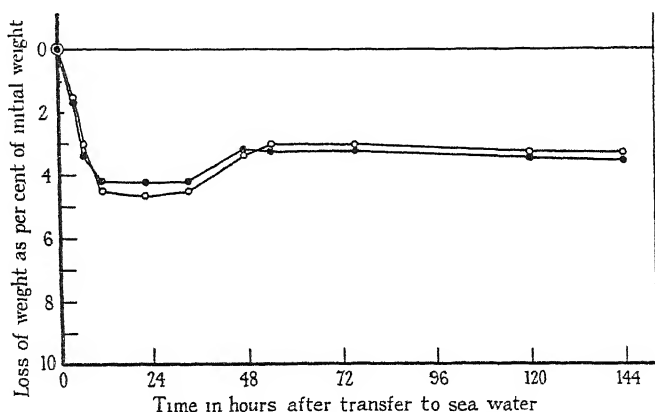


FIG. 2.—Change of weight of fresh water eels following transfer to sea water (freezing point $-1.87^{\circ}\text{C}.$). Temperature $11^{\circ}\text{C}.$ throughout the experiment.

Fig. 2 shows the weight changes of two eels following transfer from tap water to sea water; the results obtained are typical of three pairs of experiments of the same kind. The general effect, a decrease of weight, is as would be expected, but there are several features of the curves which require special notice. Each curve shows a rapid loss of weight followed by a period of little or no change, and this in turn is followed by a marked *gain*. The last part of each curve shows only the normal metabolic weight loss, and it is evident that, so far as water movement is concerned, the adaptation process is completed in about two days. Only passive response is required to explain the rapid loss of water shown in the first part of the curves, but the intervention of an active

process is indicated after about 10 hours; this process for a time balances the passive water loss and later overcomes it. Finally, active and passive forces appear to be balanced at a level where the water content of the eel is considerably less than in fresh water.

If this explanation is correct, a sufficiently prolonged narcosis should have little influence on the first part of the curve, but might be expected to delay the intervention and lessen the rate of the active process. Fig. 3 shows the effect of amytal narcosis produced by a single injection.

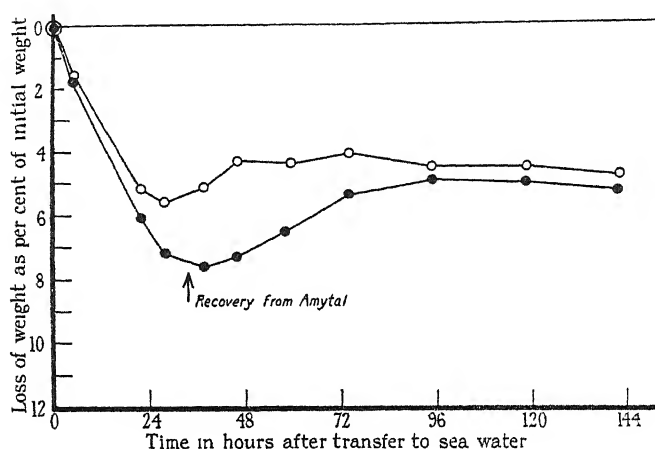


Fig. 3.—Effect of amytal on change of weight of fresh-water eels following transfer to sea water. The point marked “recovery from amytal” indicates the time when spontaneous voluntary movements were first observed. Control eel of same history but no amytal is also shown (open circles).

The expectation is fulfilled and the initial part of the curve is prolonged and the “active” process is slower. It is significant that, after the effects of the narcotic have disappeared, a steady state is attained at a level close to that of the control eel (open circles).

Fig. 4 shows the result of a similar experiment with a larger dose of amytal and an extremely prolonged narcosis. Repetitions of these experiments produced similar results.

Weight changes of eels transferred from sea water to fresh water are shown in fig. 5; the results are typical of five similar experiments. Here again there is indication of a passive process, and of the intervention of an active process, by means of which the passive movement of water is counteracted and overcome, and a steady state finally reached where active and passive processes are balanced.

The fatal effects of prevention of swallowing on the acclimatised eel in sea water and lack of effect on the acclimatised eel in fresh water have been described. Similar balloon experiments were made with eels transferred from fresh water to sea water. As expected, these eels lost weight even more

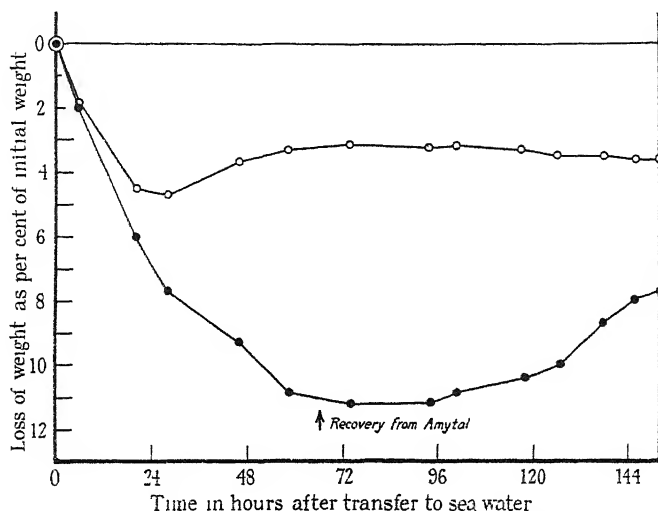


FIG. 4.—Effect of amytal on change of weight of fresh-water eel following transfer to sea water. In spite of the extremely long narcosis, this eel recovered fully and, when weighed one week after the last point shown on the graph, reached the same level of weight change as the control.

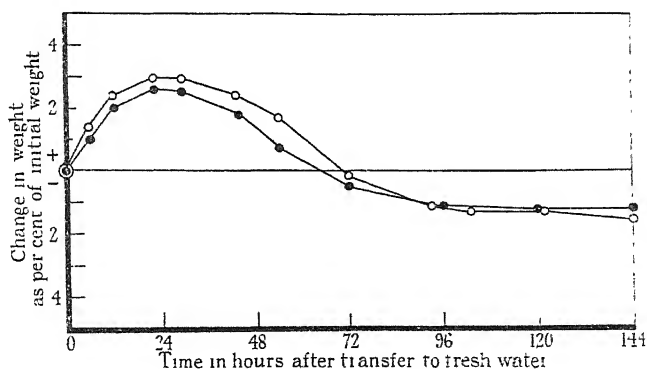


FIG. 5.—Change of weight of eels following transfer from sea water to fresh water. Temperature 9° C.

rapidly than the eel in fig. 1 and died in less than 48 hours. The vital necessity of drinking in the adaptation to sea water was demonstrated.

The converse experiment of blocking the digestive tracts of eels by means of the balloons and transferring the animals to fresh water gave the interesting

results shown typically in fig. 6. The rate of augmentation of weight (initial gain of water) was very much slowed, but, apparently, the intervention of the active process did not begin until the total weight change (water absorption) had attained a level similar to that reached by the normal animal before the active process began in it.

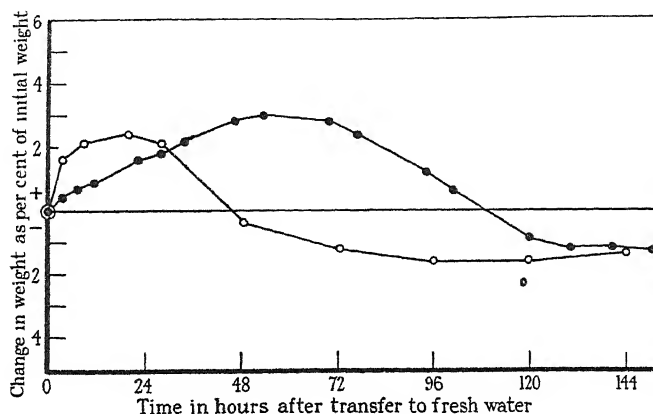


FIG. 6.—Change of weight of eels following transfer from sea water to fresh-water, and effect of blockage of digestive tract on the weight change.

The second stage in the adaptation, the active elimination of water, proceeded at the same rate in both cases, and the ultimate level reached was the same. In one case the balloon was removed immediately after the weight curve had begun to turn downward; there was no effect on the subsequent course of the weight change. The rate of adaptation of the sea water eel to fresh water is accelerated by drinking a small amount of water; this adaptation, however, proceeds perfectly well at a slower pace without this fillip.

Blood Serum Concentrations.

A serial record of the changes in concentration of the blood constituents during adaptation to sea water and to fresh water would be of great interest. Unfortunately, it is not possible to take successive blood samples for this purpose, but valuable information may be gained from the blood sera of different individuals at various stages of acclimatisation. Table I gives the results of measurements of serum chloride concentration, refractive index, and a few determinations of the freezing point depression of the serum. The last three columns in the table require some explanation.

Table I.—Measurements of freezing point depressions, chloride concentrations, and refractive indices of eel serum, together with calculated refractive increments due to salts and proteins and indicated protein percentages.

External medium.	External medium $\Delta^{\circ}\text{C.}$	Duration in medium and conditions.	Δ of blood $^{\circ}\text{C.}$	Serum chloride mg./100c.c.	Refractive index of serum at 17.5°C.	Approximate refraction due to		Indicated protein per cent.
						Salts.	Proteins.	
Fresh water	0.02	3 days	—	489	1.34879	0.00201	0.01358	7.3
"	0.02	4 days	0.63	501	1.34924	207	1397	7.5
"	0.02	4 days	0.60	480	1.34818	196	1302	7.0
Sea water	1.90	4 days	0.69	535	1.35016	219	1477	7.9
"	1.90	4 days	—	573	1.35095	234	1541	8.3
"	1.90	8 days	—	550	1.35103	225	1558	8.4
"	1.90	10 days	0.78	580	1.35058	238	1500	8.1
Fresh water	0.02	9 hours from sea water	—	537	1.34974	221	1433	7.7
"	0.02	" "	—	520	1.34930	214	1396	7.5
"	0.02	24 hours from sea water	—	505	1.34823	208	1295	6.9
"	0.02	" "	—	ca. 500	1.34789	206	1263	6.8
Sea water	1.87	9 hours from fresh water	—	560	1.35052	230	1502	8.1
"	1.87	24 hours from fresh water	—	573	1.35109	234	1555	8.4
Fresh water	0.02	Balloon, 9 hours from sea water	—	558	1.35036	230	1486	8.0
"	0.02	" "	—	564	1.35000	232	1448	7.8
"	0.02	Balloon, 24 hours from sea water	—	526	1.34952	216	1416	7.6

The refractive index of the serum is made up of three additive components : (1) that due to the refractive index of the water, which is 1.33320 at 17.5°C. ; (2) the part contributed by non-protein substances, due almost entirely to the salts ; (3) the part contributed by proteins. If an estimate of the total salts is available, and the total refractive index is known, the part contributed by serum protein, and therefore the protein concentration, can be obtained.

Analyses for Cl, Ca, Mg and SO_4 in eel serum showed relative proportions of these substances which are in substantial agreement with Smith's (1929) more complete analyses of sera from other fish species. Other data on mineral distribution in eels as compared to other vertebrates (Katz, 1896) permitted an approximate tabulation of the ionic proportions of the mineral constituents of eel serum. The molar proportions thus obtained are : Na, 48.2 ; K, 1.4 ; Mg, 0.3 ; Ca, 1.2 ; Cl, 42.6 ; SO_4 , 0.6 ; PO_4 , 1.2 ; CO_2 , 1.7. From these figures and from values for the specific refractions of the various substances fig. 7 was constructed. The excess of mineral cation is taken as albuminate

and is reckoned with protein. The comparison lines for salts are calculated from Landolt-Börnstein tables after Wagner; the line for serum proteins is from the values of Adair and Robinson (1930) for serum albumins and globulins.

The values in Table I in the column "approximate refraction due to salts" are calculated from the upper limit of the shaded area in fig. 7, which relates the chloride concentration to the probable part of the total refraction contributed by non-protein substances.

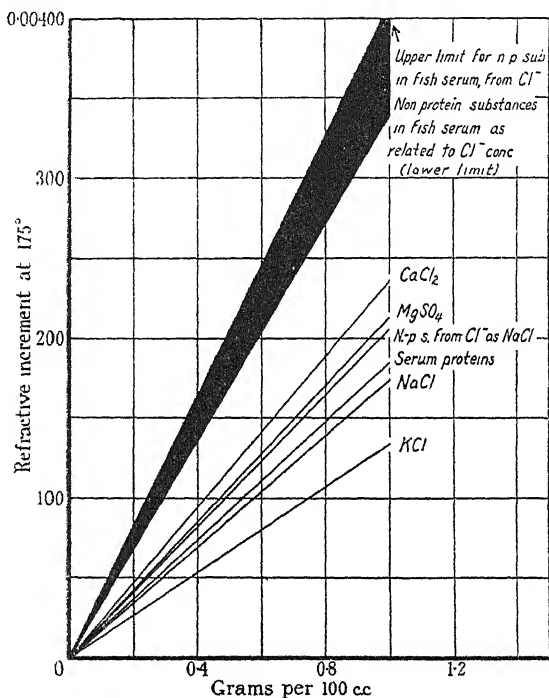


FIG. 7.—Graph for the approximate calculation of serum protein from the chloride concentration and the total refractive index. The values are for refraction in aqueous solution at 17.5° C. See text.

The figures for serum concentration corroborate the conclusion of earlier workers (*e.g.*, Quinton, 1904; Dakin, 1908; Schmidt-Nielsen, 1923; Duval, 1925) that the proportion of water in the serum is higher when the eel is living in fresh water than when it is in sea water. During acclimatisation the water concentration is intermediate and changes more slowly in eels which are unable to swallow than in normal eels. The determinations are few, but they make it highly improbable that the water concentration behaves as does the total water content of the eel during acclimatisation. Moreover, it appears that the chloride and protein concentrations are at least roughly proportional, as

will be seen from fig. 8, in which Cl concentrations are plotted against protein concentrations in the same sera.

There is a suggestion that the chloride concentration tends to change less rapidly than protein in the course of adaptation; this would be expected from the action of the tissues as a buffer against chloride change (Wahlgren, 1909; von Hosslin, 1909).

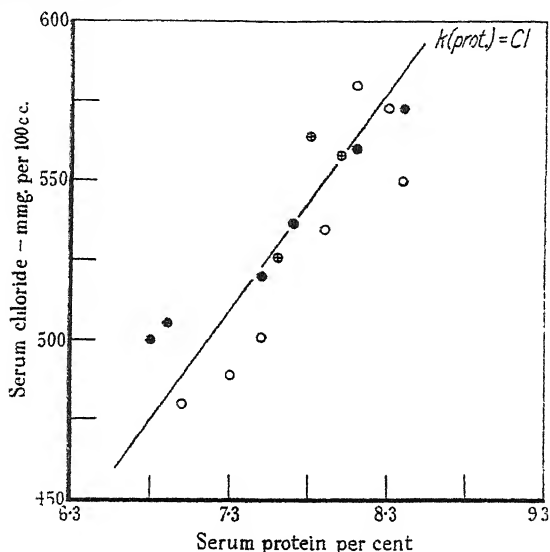


FIG. 8.—Relation between chloride and protein concentrations in eel serum. The line drawn is that of the equation: [protein] \times constant = [Cl]; the constant being taken as 70 with the units employed here. Open circles, fully acclimatised eels; solid circles, eels in course of adaptation; crossed circles, “balloon eels.”

The freezing point depressions reported here for sera from acclimatised eels are compared with earlier determinations in Table II.

Table II.—Freezing Point Depression of Eel Serum.

Eels in fresh water.		Eels in sea water.	
Authority.	Mean freezing point depression.	Authority.	Mean freezing point depression.
	° C.		° C.
Fredericq (1905)	0.64	Dakin (1908, a)	0.66
Dakin (1908, a)	0.57	Dakin (1908, a)	0.74*
Dakin (1908, b)	0.58	Dakin (1908, b)	0.63*
Portier and Duval (1922)	0.63	S. and S. Schmidt-Nielsen (1923)	0.68
S. and S. Schmidt-Nielsen (1923)	0.56	Duval (1925)	0.71
Duval (1925)	0.62		
Present determinations	0.61	Present determinations	0.73

* Denotes single determinations under different conditions of capture.

Comparison of chloride concentrations is less satisfactory for the reason that the older methods were only relatively accurate. For the present purpose, however, relative values will suffice and these are compiled in Table III. The value reported for the eel in fresh water is taken as 100 per cent. in each case.

Table III.—Relative Chloride Concentrations of Eel Serum.

Authority.	Mean for eels from fresh water.	Mean for eels from sea water.
Quinton (1904, <i>a</i>)	100	140
Dakin (1908, <i>a</i>)	100*	132*
Duval (1925)	100	116
Present determinations	100	115
	107	115*
	(9 hours from sea water)	(9 hours from fresh water)
	103	117*
	(24 hours from sea water)	(24 hours from fresh water)

* Denotes single determinations.

The rather big differences between Cl concentrations of fresh water and sea water eel sera reported by Quinton and Dakin may have been due to insufficient precautions in handling the animals, since it is known that damage to the mucous coat brings about results of this kind (Regnard, 1891, p. 438; Schmidt-Nielsen, 1909; Portier and Duval, 1922; Duval, 1925).

The protein percentages indicated in Table I are similar to those found in mammals and point to a colloid osmotic pressure of about 25 mm. Hg.† Recent determinations (Keys and R. M. Hill, unpublished work) gave 20 mm. Hg for the average colloid osmotic pressure of serum from fresh-water eels. For eels long starved (32 to 70 days) lower values were found, but there was a consistent difference between eels in fresh water and in sea water; the averages were 8.5 mm. Hg in fresh water and 11 mm. in sea water. Prevost and Dumas (1821) found the total solid in eel blood to be 15.4 per cent., from which Schulz and von Krüger (1925, p. 1129) calculate the solid of the serum to be

† This value may seem improbably high since fish blood pressures are generally low (Schoenlein, 1895; Brünings, 1899; Hyde, 1908; Lyon, 1925; Lutz and Wyman, 1932), and glomerular filtration requires that the hydrostatic pressure exceed the colloid osmotic pressure by a considerable margin. However, the familiar low values (20 to 40 mm. Hg arterial pressure) were derived mainly from studies on elasmobranchs; Greene (1904) reported an average blood pressure of 74 mm. in the ventral aorta and 53 mm. Hg in the dorsal aorta of the salmon in fresh and brackish water. Greene found somewhat lower values in salmon in sea water (where very little urine is formed). Finally, the possibility of direct secretion (Marshall, 1930) must be borne in mind.

10 per cent. on the basis of data for corpuscular volumes (Hoffmeyer, 1907; Malassez, 1872); from the same data my calculations indicate about 8 per cent. solids in the serum. Halliburton (1886) reported the plasma of the (fresh water) eel to contain 6.73 per cent. total protein.

Discussion.

Indications of weight-change curves similar to those reported here for adaptation are contained in earlier papers. Quinton (1904) transferred eels from sea water to fresh water and obtained curves similar to those given in fig. 5, but the experiments were discontinued before a steady state was reached. His chloride analyses show a regular and progressive dilution of the blood. Working with the partly euryhaline fish *Fundulus heteroclitus*, Sumner (1905), "found in most instances that the endosmotic flow of water had ceased and that a secondary *decrease* in weight had ensued within 1 or 2 days after transfer to fresh water. . . . Fishes of this species commonly did not die for a considerable number of days."

Duval (1925, p. 295) believed that eels attain a new steady state in 2 hours following a change in the external salinity, but his evidence is hardly conclusive and is opposed by the data of all other investigators.

Space does not permit a detailed discussion of work on other fishes (see Dekhuyzen, 1904, 1905; Dakin, 1908, *a, b*; Scott, 1916; Schmidt-Nielsen and Schmidt-Nielsen, 1923; Duval, 1925; Schlieper, 1930; and Smith, 1932); all evidence points to the essential normality of the eel as a fresh-water fish in fresh water and as a marine fish in sea water. The same may be said of the other euryhaline teleosts so far as they have been investigated. The eel in sea water produces, similarly to the normal marine teleost, a very small amount of urine which is isotonic or hypotonic to the blood, and in fresh water it, like the fresh water teleosts, produces large quantities of very dilute urine (Rodier, 1899; Dekhuyzen, 1904, 1905; Bottazzi, 1906; Smith, 1930; Marshall, 1930; Smith, 1931, personal communication).

The blood of the euryhaline species in sea water is practically undistinguishable in osmotic concentration from the blood of the stenohaline marine forms. Both types possess the ability to extract water from sea water, and it appears that the mechanism is the same in both cases—sea water is swallowed and absorbed from the gut and the excess chloride secreted by the gills (Smith, 1930; Keys, 1931; Bateman and Keys, 1932; Smith, 1932). The euryhaline fishes and the fresh water teleosts possess kidneys capable of copious

filtration and effective salt conservation (Nash, 1931 ; Smith, 1932). These kidneys are characterised by large glomerular surfaces as compared with the marine fishes, in some of which glomeruli are totally lacking (Audigé, 1910 ; Marshall and Grafflin, 1928).

It would appear that the fish kidney is capable of regulating only toward a high osmotic pressure of the blood ; regulation toward a low osmotic pressure of the blood is performed exclusively by a special mechanism in the gills, and this mechanism is absent in the fresh-water fishes. Adaptation over wide ranges of salinity requires both mechanisms, but passive responses precede the intervention of an active mechanism. Accordingly, we should expect the weight-change curve of a euryhaline fish to be approximately logarithmic during the first period following a change in the external salinity. This was found to be so with the eel ; the weight-change curve is roughly logarithmic from about the second hour after transfer until about three hours before the intervention of the active process is unmistakable.

Comparison of fig. 2 with fig. 5 suggests that the adaptation from fresh water to sea water may require less time than the reverse ; this was, in fact, almost the invariable result, and there does not seem to be any ready explanation. The conclusion of Lucké and McCutcheon (1927), "that cells are more permeable to water when the osmotic concentration of the medium is high than when it is low," helps one to understand the difference between the "passive" portions of the weight-change curves, but the chief difference is found in the times from the intervention of the "active" process to the attainment of the steady state.

Weight-change curves of the kind presented here seem to be characteristic of the euryhaline fishes. Sumner (1906, p. 77) obtained results of this nature with the chinook salmon (*Oncorhynchus tshawytscha*), though sufficient weighings to construct complete curves were not done. The experiments of Gueylard (1924) on the stickleback show very clear weight-change curves of this sort. The strictly stenohaline species never show anything like these recovery curves, but graphs of their weights following abrupt transfer from sea water to fresh water and *vice versa* resemble the "passive" portions of the curves for the eel.

The role of serum colloids in the adaptive process is obvious ; if water is withdrawn from the blood by passive diffusion, the resulting increase in colloid osmotic pressure will reduce the glomerular filtration and the urinary water loss ; the reverse effect will take place following the transfer of the fish from a concentrated to a dilute environment.

The principal passive processes in adaptation to changed salinity are reducible to diffusion of water and salts through more or less semi-permeable membranes; swallowing and the utilisation of stores of salt and water in the tissues can effect only the rate of attainment of the final state. In the adaptation from sea water to fresh water and the osmotic regulation in fresh water after acclimatisation, the kidney is the responsible active organ. In sea water, however, and in the adaptation from fresh water to sea water, sea water is ingested and absorbed from the gut, and the excess salt is secreted out from the gills (Keys, 1931), which bear the burden of osmotic regulation. In both cases of adaptation the regulatory organs begin functioning effectively only after a considerable change in concentration of the blood has taken place. Moreover, it is concluded that these organs continue functioning only under constant stimulus of high and low blood concentrations respectively, with the result that, even in the eel, osmotic regulation is not perfect and the blood concentration reflects faintly the external concentration.

It is a great pleasure to be able to record my obligations to Professor August Krogh and his staff in Copenhagen and to Professor Barcroft, F.R.S., in Cambridge. I am especially grateful to those who gave laboratory help and collaboration in the various experiments on osmotic regulation in the eel, reported here and in previous papers: Dr. J. B. Bateman, Dr. R. M. Hill, Mr. E. N. Lange, and Mr. E. N. Willmer.

Summary.

Experiments have been performed with eels in sea water and in fresh water, and the water movements following abrupt change of external salinity were studied. By various experimental procedures a partial separation was made of active and passive forces involved in the adaptive process.

Studies were made of blood concentrations during and after the completion of the adaptive process, and these results are correlated with those obtained in the experiments on water balance and with the properties of the branchial secretory mechanism.

Evidence is assembled from which it appears that the euryhaline teleost may be considered to be a typical fresh-water fish in fresh water and a typical marine fish in sea water.

It is shown that the changes in water content of the eel during adaptation to changed salinity are not paralleled by changes in water or osmotic concentration of the blood. The curve of weight change (water content) during

adaptation shows a period of passive water movement, a temporary steady state at a level very different from the initial level, a movement of water in the reverse direction from that which would be expected from the concentration gradient, and a final steady state in which the water content of the animal is close to the original water content before the adaptation.

Data are presented which indicate that the colloid osmotic pressure of the serum behaves like the total osmotic pressure. The effect of this on the urinary output is discussed.

The role of water swallowing in the adaptation, and in normal life, in sea water and in fresh water is shown experimentally.

It is shown that, so far as evidence is available at present, the phenomena observed in the eel are typical of the euryhaline teleosts, and that there are two primary mechanisms involved in the active regulation of osmotic concentration. The kidney conservation of salt and elimination of large amounts of water is shared with the stenohaline fresh-water fishes; the elimination of excess salt and conservation of water by the gills appears to be common to the stenohaline marine teleosts and the euryhaline forms like the eel.

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*The Development and Morphology of the Gonads of the Mouse.—IV.
The Post-Natal Growth of the Testis.*

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INTRODUCTION.

The early development of the gonads of the mouse, including the differentiation of the ovary and testis, was described by Brambell (1927) in the first part of this series, together with the subsequent development of the ovary. It was intended to describe in a subsequent paper the development of the testis from the time of its differentiation until maturity, but examination of an extensive series of embryonic testes did not yield results of sufficient importance to require publication, since the paper by Agdhur (1927), dealing with this subject, had appeared in the meantime. The present paper deals with the development of the testis from birth until long after sexual maturity is attained, and is, therefore, the logical completion of the series begun in 1927.

This paper deals chiefly with the sizes of the testes and their measurable components in relation to the age and the cleaned-body weight. The results, so obtained, are correlated with the more clearly defined histological phases, but no attempt is made to describe systematically the post-natal histology or cytology of the testis.

The work has been executed with a view to providing data of the normal growth of the testis in the mouse, such as Donaldson (1924) has provided for the rat, which should serve as controls for experimental work. In particular an attempt has been made to treat separately the growth of two component tissues of the testis—the spermatie tubules and the intertubular tissue. We define the “intertubular” tissue as all tissues within the tunica albuginea other than the spermatie tubules. It therefore includes connective tissue, blood-vessels, lymph-spaces, etc., as well as the so-called “interstitial” cells. It was found impossible to measure accurately the amount of interstitial cells present.

MATERIAL.

Albino mice were employed because they provide suitable material for subsequent experimental work and because they have no restricted breeding season or correlated seasonal enlargement in the reproductive organs.

All the animals employed were derived from the colony in this department, which is an off-shoot of the in-bred colony employed by Parkes (1926). We would like to take this opportunity of expressing our thanks to Dr. A. S. Parkes for providing the animals from which our colony has been raised. Care was taken to insure that the diet was uniform and adequate.

Litters of mice are usually born during the night and are consequently some hours old when first observed in the morning. They were considered to be half a day old on the morning after birth in all cases.

The expenses of this research were defrayed in part by a grant from the Government Grant Committee of the Royal Society to one of us (F.W.R.B.), for which we wish to express our thanks.

TECHNIQUE.

(a) *Method of Weighing.*—The cleaned-body weight was employed in preference to the entire-body weight as being more constant and not affected by the state of distention of the gut and bladder. The stomach and intestine were removed for this purpose, after ligaturing the coeliac artery and the portal vein, and the bladder was evacuated.

The testes were weighed separately in weighing bottles, after the removal of the epididymis and of all fat from each. The weights were checked by taking the combined weight of the two testes after transferring one testis to the bottle containing the other. This involved a slight loss in weight through loss of moisture condensed on the first bottle.

(b) *Histological Treatment.*—The testes were immersed in fixative as soon as possible after weighing. In no case did more than 15 minutes elapse between killing and immersion in the fixative. Bouin's fluid was always employed, except that for the larger testes the modified alcoholic fluid, in which a saturated solution of picric acid in 70 per cent. alcohol is substituted for the saturated aqueous solution, was used. This modification was necessary to ensure rapid penetration, since it was not possible to cut the tunica albuginea and thus hasten penetration, as this would have affected the size relations of the tissues. Care was taken to standardize so far as possible the fixation and subsequent treatment of the testes, since small variations in technique introduce an uncontrollable error in subsequent measurements through producing different amounts of shrinkage in the tissues. Complete serial sections, cut transversely at 10 μ thick, were made of one testis of each animal, and were stained with Ehrlich's hæmatoxylin and eosin.

(c) *Measurement of Tissues*.—Drawings were made, at a magnification of 100 diameters, by means of a Leitz micro-projection apparatus, of the largest transverse section of each testis. The diameter of each tubule and the total number cut across was determined from these drawings. Since many tubules are cut tangentially in a transverse section and appear elliptical the diameter of each was taken as the greatest width at right angles to the long axis of the ellipse. The mean diameter of all the tubules in the greatest transverse section, measured in this way, was determined for each testis.

The total area of the spermatic tubules and of the intertubular tissue was also estimated from these drawings. This was done by cutting out and weighing the drawing, then cutting out all the tubules, weighing them and the residue of the paper. The weights were subsequently converted to estimates of area by employing as standard the weight of a known area of the same paper, which was assumed to be uniform.

(d) *Treatment of Results*.—The results obtained are, with the exception of the relation of the weight of the right testis to that of the left, presented in the form of scatter diagrams. These bring out the relevant points concerning the growth changes, and possess at the same time the advantage of rendering all the data available for statistical analyses. It was found impracticable to fit curves represented by polynomial terms since the data were unequally spaced.

The data of the relative weights of the right and left testes were treated as a straight line regression.

We are indebted to Dr. A. S. Parkes, Dr. J. Wishart and Dr. F. G. Soper for advice and criticism.

OBSERVATIONS.

Post-natal Growth in Body-weight.

During the course of the researches on the growth of the testis data of the cleaned-body weight and age of one hundred male mice, ranging from newly born to animals 300 days old, were accumulated. These data, fig. 1, are sufficient to determine the general form of the growth in cleaned-body weight on age, which has an important bearing on the interpretation of the changes described in the testis. It is not the purpose of this paper to deal with growth in body-weight as such or to offer any theoretical interpretation of the form of the growth-curve, for which purpose more extensive data would be desirable.

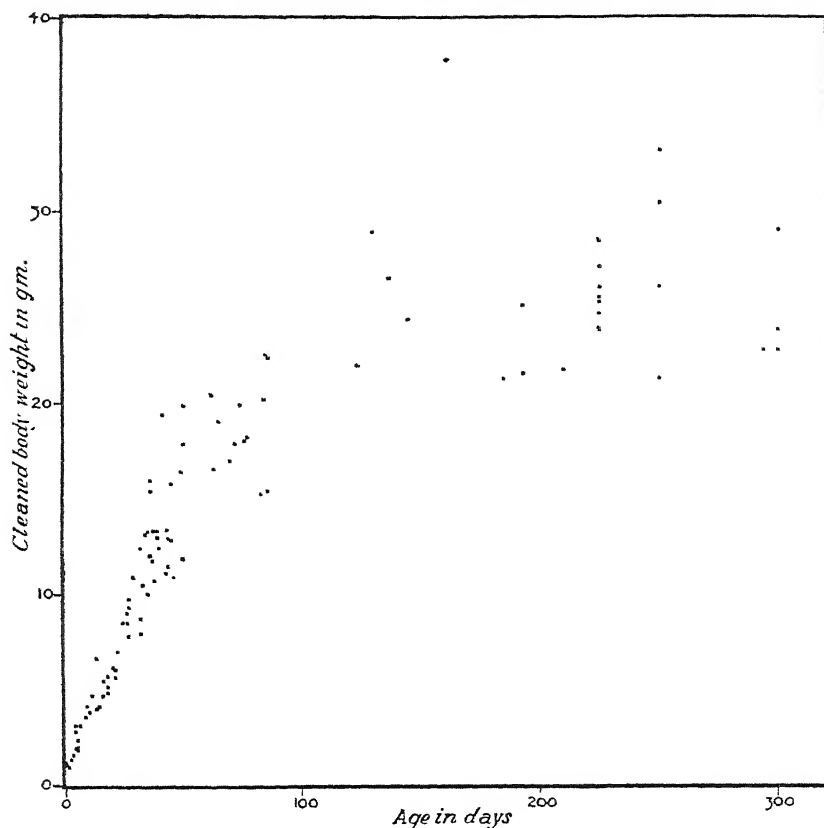


FIG. 1.

Post-natal Growth of the Testes.

(a) *Relation of Weight of Testes to Cleaned-body Weight.*—The data available consisted of accurate weights of 115 animals ranging from 1 to 33 gm. cleaned-body weight. They are given in fig. 2 in the form of a scatter diagram.

(b) *Relation of Weight of Testes to Age.*—The data, consisting of the weights of the testes of 101 animals of known age ranging from birth to 300 days, are given in fig. 3.

(c) *Relation of the Weight of the Testes expressed as a Percentage of the Cleaned-body Weight to Age.*—The data, derived from the same 101 animals as in section (b) are given in fig. 4. It can be seen that the percentage weight of the testes rises rapidly from about 10 to 40 days old, when the maximum is attained. Subsequently the percentage weight of the testes remains constant throughout life at a value of approximately 0.85 per cent. body weight.

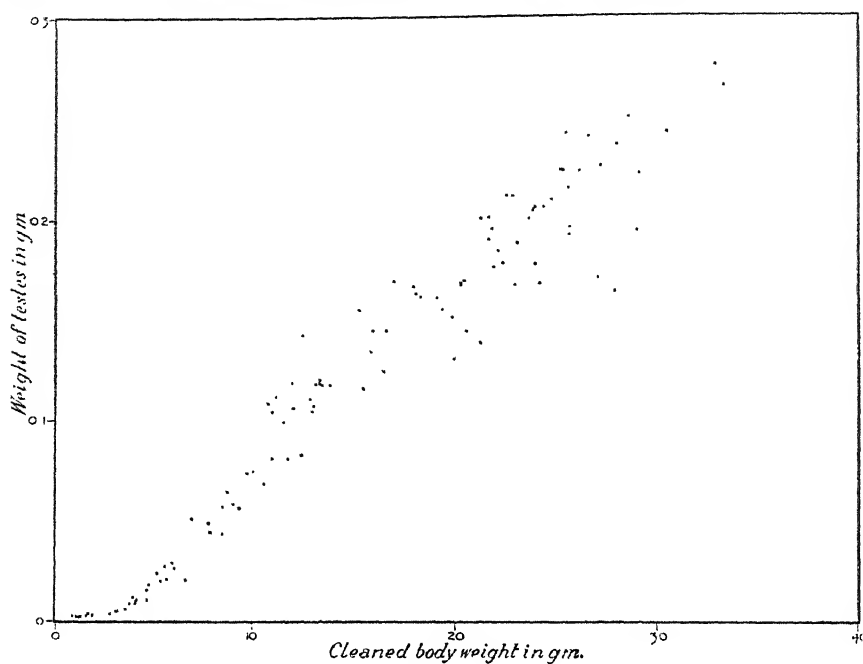
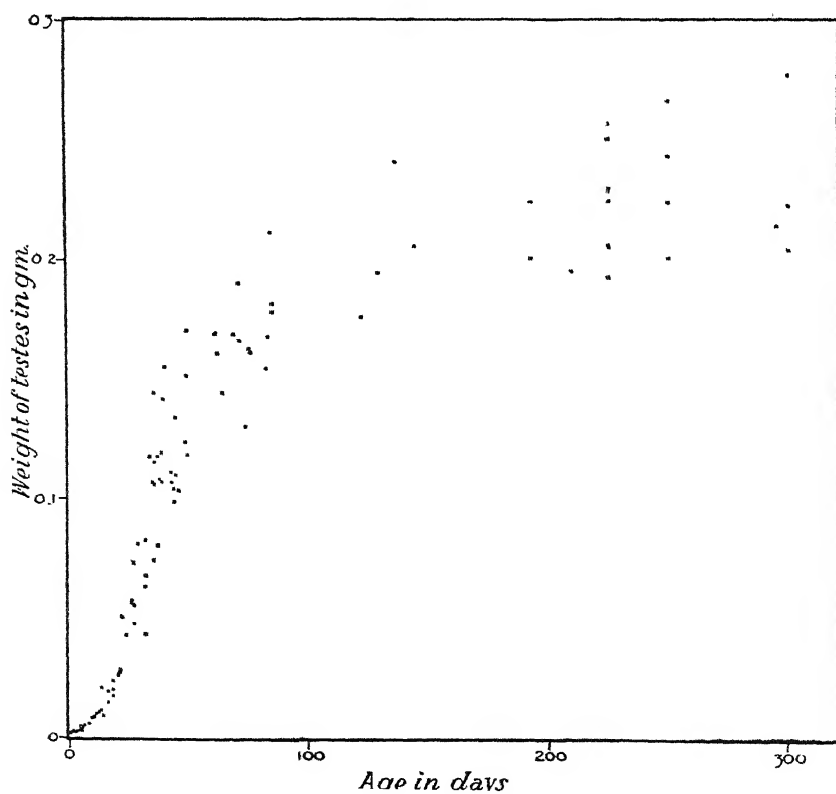


FIG. 2.



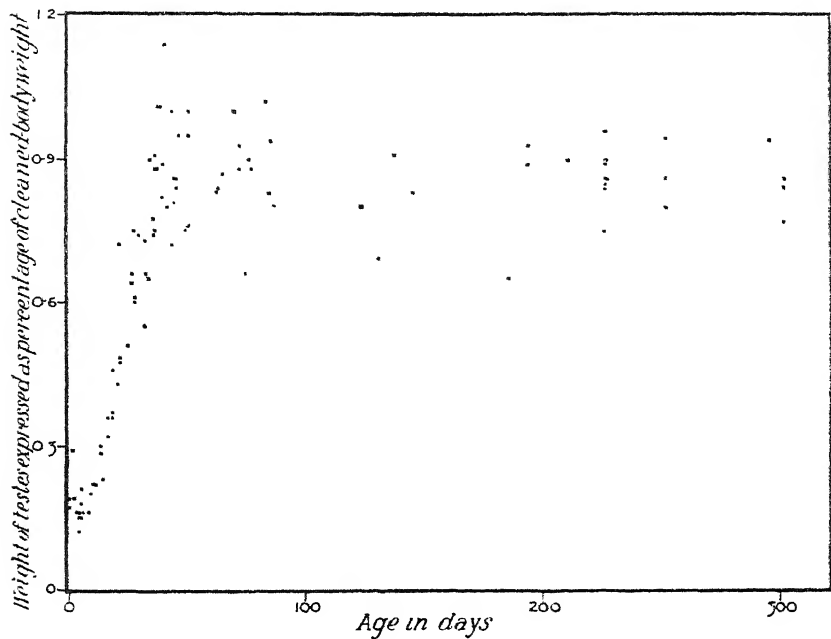


FIG. 4.

(d) *Size of Right and Left Testes.*—Comparison of the weights of the right and left testes of 92 pairs showed :—

Right > left	76
Right = left	3
Right < left	13

The data were fitted with a straight regression line for the weight of the right testis on the weight of the left testis. The regression is expressed by the formula $Y = 1.034x$, where Y = weight of the right testis and x the weight of the left testis in grams. Testing the significance of the difference between this regression coefficient and the hypothetical value, *i.e.*, $Y = X$, where the two testes are of equal weight $t = 7.57$ and $n = 90$. Entering Fisher's (1930) table of t with these values P is found to be less than 0.01 and the difference between the two regressions must be considered decidedly significant. The regression line is represented by a continuous line in fig. 5, and the hypothetical regression line, if the weights of the two testes tended to equality, is represented by the dotted line.

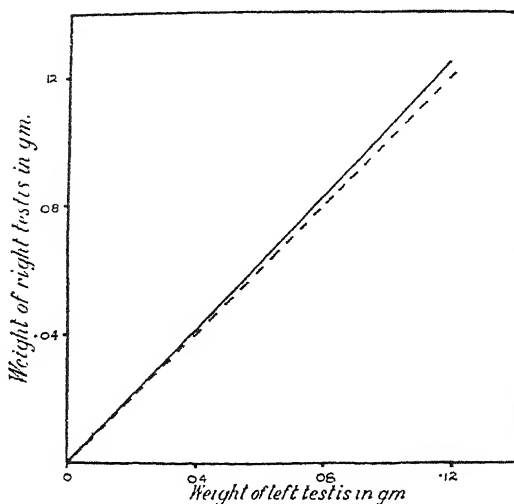


FIG. 5.

Post-natal Growth of the Component Parts of the Testis.

(a) *Increase in Diameter of the Spermatic Tubules.*—The data consist of the mean diameters of the tubules in the testes of 80 animals of known ages from

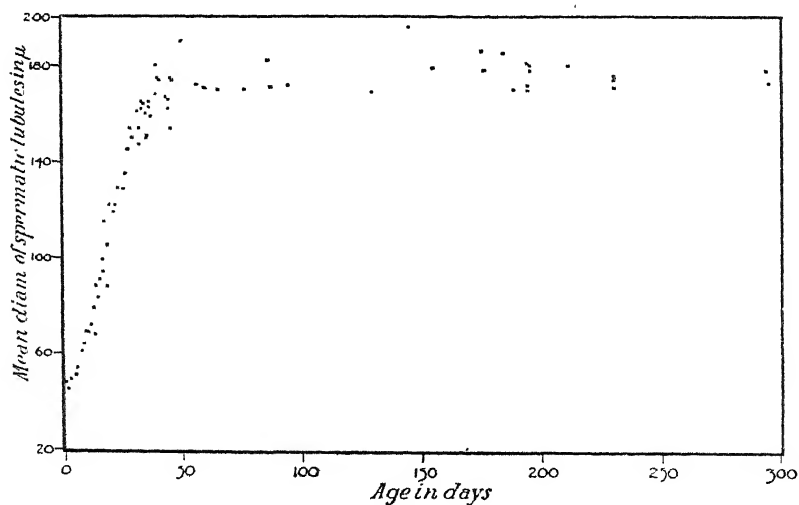


FIG. 6.

birth to 300 days. The mean diameter of all the tubules in a single transverse section across the middle of one testis was taken in each case as the diameter of the tubules in that animal. The data are represented in fig. 6.

It can be seen that there is little or no appreciable increase in the diameter of the tubules after the 40th day.

(b) *Number and Area of Spermatic Tubules in Transverse Sections.*—The number of spermatic tubules cut across in a transverse section of the testis appears to remain approximately constant from shortly after birth until maturity. This is shown by the correlation table.

Correlation Table.

Number of spermatic tubules in transverse section.	Age in days.						
	10-19.9.	20-29.9.	30-39.9.	40-49.9.	50-59.9.	60-69.9.	70 <.
250-274.9	1	3	—	1	—	—	—
225-249.9	—	3	3	3	—	—	1
200-224.9	4	1	7	3	—	2	2
175-199.9	2	1	3	1	2	—	1
150-174.9	2	—	—	—	—	—	—
125-149.9	—	—	1	—	—	—	—
100-124.9	1	1	—	—	—	—	—

The constant number of spermatic tubules in a transverse section, which implies also that they do not become more convoluted, since this would result in a consequent increase in the number of times they would be cut across, is also brought out by the data of the total areas of the spermatic tubules in transverse section. These data are available for 62 animals, ranging in age from 10 to 300 days, in which the mean diameter of the spermatic tubules was known. The total area of the spermatic tubules is plotted against the square of the mean diameter for 62 animals in fig. 7. It is obvious that the data tend to fall on a straight line, thus showing that the total area of the spermatic tubules in a transverse section of the testis is a constant function of the square of the mean diameter. Expressed in other words the number of tubules cut across in a transverse section of the testis is constant from 10 to 300 days old.

(c) *Area of the Intertubular Tissue.*—The data consist of measurements of the total area of intertubular tissue in the largest transverse section of one testis of each of 56 animals of known ages from 10 to 200 days. These data are given in fig. 8, and indicate a gradual falling off in the rate of increase of the area of the intertubular tissue with increasing age.

(d) *Relative Area of Tubular to Intertubular Tissue.*—The data, fig. 9, consist of the area of the intertubular tissue divided by the area of the spermatic tubules

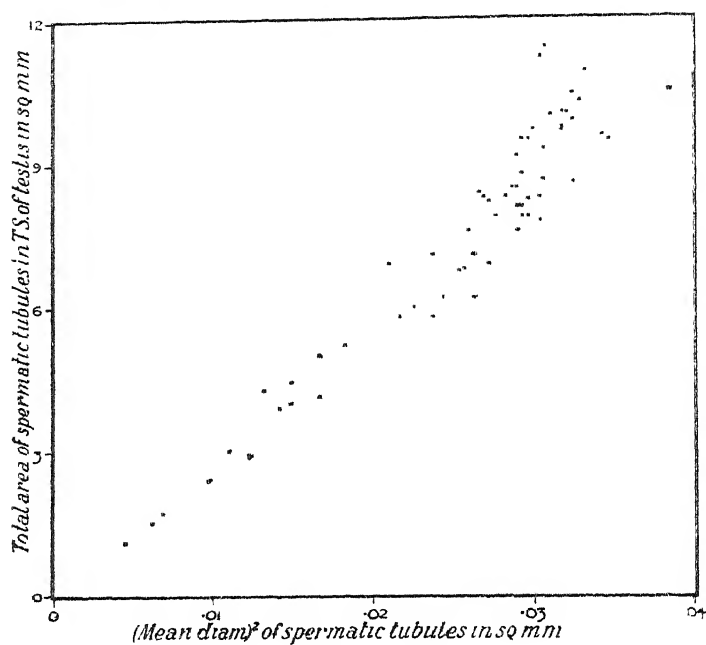


FIG. 7.

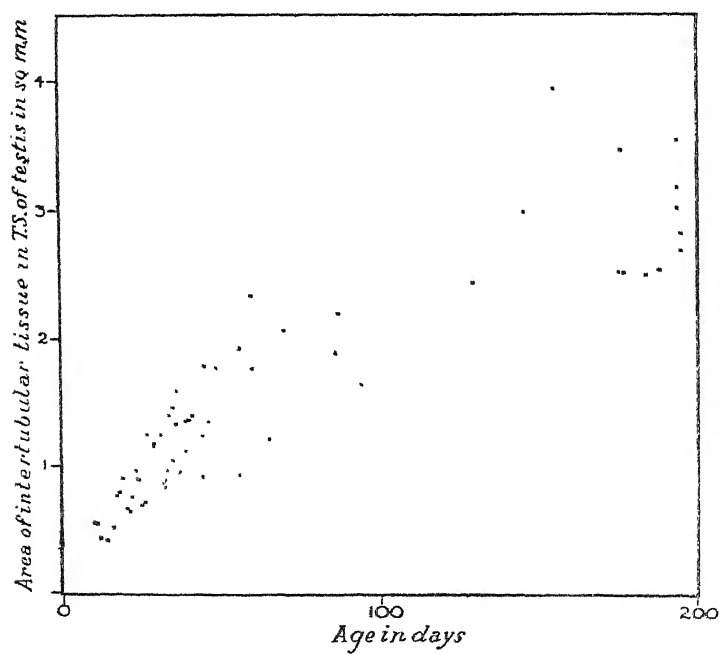


FIG. 8

in 62 animals between 10 and 300 days old. It can be seen that the relative area reaches a minimum at about 30 to 40 days old, and subsequently rises to a higher level. Small inequalities in the histological technique probably result in differential shrinkage of the tubular and intertubular tissue and account for the wide spread of the data.

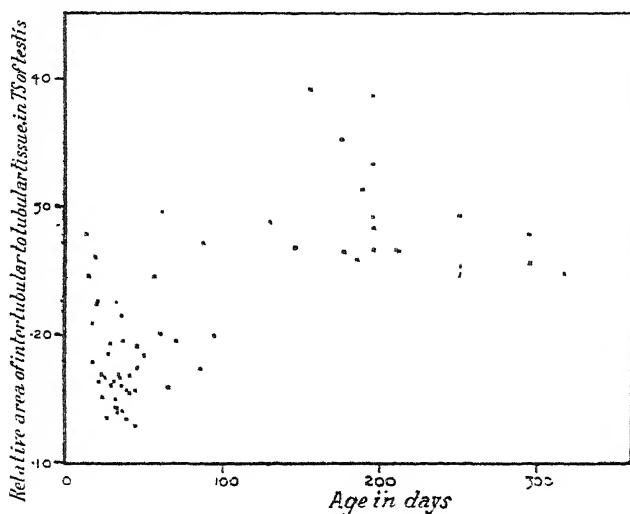


FIG. 9.

Spermatogenesis in Relation to Age.

The testes of 60 mice, ranging from birth to maturity, were examined histologically with a view to determining the time relations of the more salient points in the process of spermatogenesis. For this purpose clearly defined phases, such as pachynema, the maturation spindles, spermatids and ripe spermatozoa, were chosen and the ages at which cells in these stages first appeared and reached a maximum number were determined. The material employed consisted of the testes of one or more animals at daily age intervals from birth to 45 days old, with others at 50, 60 and 76 days old, and two animals upwards of one year old.

The spermatogenic tubules in the new-born mouse are solid and the contained germ-cells are spermatogonia and primary spermatocytes. Some of the spermatogonia are in mitosis and the primary spermatocytes are in the deutobroch stage. Spermatocytes with pachytene nuclei appear early and reach a maximum on the 13th-14th day when they are very numerous and form a layer three or four cells thick in the tubule. Subsequently, though always

present, pachytene nuclei are less numerous. A lumen first appears in some of the tubules on the 13th day, and in a day or two is present in all. After this time spermatogenesis appears to proceed more actively in some tubules than in others. Soon heterotypic spindles appear and the first maturation division takes place. On the 18th day some of the tubules exhibit heterotypic spindles while in others the first maturation division is completed and secondary spermatocytes are present. The secondary spermatocytes do not enter upon a resting stage, so that the second maturation division follows soon after the first, and spermatids are found in the tubules by the 20th day. At this time the seminal epithelium consists of a single row of spermatogonia next the wall of the tubule, then a row of primary spermatocytes followed by a layer of secondary spermatocytes, two or more cells thick, and finally a row of spermatids next to the lumen.

The first indications of spermateleosis, or the metamorphosis of the spermatids into spermatozoa, are discernible by the 21st day, and by the 30th day this process is taking place in many cells. At the age of 33 to 35 days immature spermatozoa with tails are present attached to the Sertoli cells by their acrosomes, but still retaining the cytoplasm of the spermatid which is subsequently sloughed off. Immature spermatozoa are very numerous and some are in process of sloughing off the residual cytoplasm by the 40th day. Mature spermatozoa, free in the lumen of the tubules, are found first at the age of 42 days. Testes at 45 and 50 days old showed large numbers of mature sperms together with a further lot of spermatids in spermateleosis. At 60 days sperms were present in the lumina of the efferent tubules but may have been present here a few days earlier. Spermatogenesis was still proceeding actively in the testes over a year old. No marked wave of degeneration in the germ-cells, such as has been recorded in a number of other mammals shortly before puberty, was observed at any stage between birth and maturity in the mice examined.

Discussion.

The increase in weight of the two testes is given both in relation to cleaned-body weight and to age. It can be seen that the most rapid increase in the weight of the testes occurs at a body weight of 10 to 12 gm., fig. 2, and at an age of 30 to 40 days, fig. 3. Further the rate of increase in the weight of the testes drops off rapidly after about 60 days. The diagram of the percentage body weight of the testes on age, fig. 4, brings out an interesting relation. The data from 10 to 40 days approximate to a straight line relationship indi-

cating a constant rate of increase in the percentage body weight of the testes over this period. Subsequently the percentage body weight of the testes appears to remain constant at approximately 0.85 per cent. body weight.

The fact that the right testis tends to be significantly heavier than the left is clearly shown in fig. 5.

The diagram of the mean diameter of the spermatic tubules on age shows a maximum rate of increase at about a fortnight old. It can be seen from fig. 6 that the curve begins to flatten out at 40 days, and that, subsequently, there is little or no perceptible increase in the diameter of the tubules. It is remarkable that the mean number of spermatic tubules cut across in a transverse section through the middle of the testis appears to remain approximately constant from 10 days old, although the actual number is variable within fairly wide limits. This is also shown by the fact that data of the total area of the spermatic tubules in single transverse sections of the testis plotted against the square of the mean diameter fall on a straight line, fig. 7. The total area of the spermatic tubules in a transverse section is, in fact, a constant function of their mean diameter.

The increase in the area of intertubular tissue with age is shown in fig. 8. We have defined the intertubular tissue as all the tissue within the tunica albuginea other than the spermatic tubules. It is thus an artificial conception, including blood vessels, lymph spaces, connective tissue, etc., together with the so-called "interstitial" cells. Technical difficulties rendered it impossible to arrive at an estimate of the area of the "interstitial" tissue alone with sufficient accuracy. The relative importance of the spermatic tubules and the intertubular tissue as components of the testis is brought out by fig. 9 in which the values of the area of intertubular tissue divided by the area of the spermatic tubules are plotted against age. It can be seen that a minimal value is reached at 30 to 40 days and rises subsequently. It is perhaps significant that Kasai (1908) found two maxima in the growth of the "interstitial" tissue in the human testis respectively at the 7th month of foetal life and at puberty.

Considering the more characteristic and clearly defined phases of spermatogenesis it can be seen that primary spermatocytes are already present in the testis of the new-born mouse. The number of nuclei in pachynema reaches a maximum on the 13th or 14th day. Simultaneously with the appearance of the maximum number of pachytene nuclei, a lumen first appears in the spermatic tubules, which were previously solid. Spermatids are found first on the 20th day and mature spermatozoa free in the lumen of the tubule on the 42nd day.

Spermatozoa were found in the efferent tubules on the 60th day, but may have been present there a few days earlier.

Degeneration occurs among some of the germ-cells in some tubules and many testes but no general wave of degeneration occurs at any time after birth, such as has been described in a number of mammals by various authors.

The time-relations of spermatogenesis described above coincide in a remarkable manner with those recorded by Allen (1918) in the albino rat. This author found primary spermatocytes appearing in the tubules on the 7th to 10th day. These reached the pachytene stage by the 14th day and simultaneously the lumen first appeared. He found the first crop of spermatozoa in the tubules on the 37th day. Hewer (1914), however, also working on rats, obtained very different results, finding for the first time primary spermatocytes at $3\frac{1}{2}$ weeks, a lumen at 7 weeks, spermatids at 8 weeks, spermatozoa at 9 weeks and at 10 weeks a second crop of spermatozoa in the testis and the first crop in the epididymis. Allen's results, although in marked contrast to Hewer's, conform very closely to our results on the mouse. Moreover, he employed only rats of the standard strain of the Wistar Institute, and thus probably obtained reliable results. We employed also an inbred strain of mice, thus obtaining greater uniformity than would be possible with animals of different strains. We lay great stress on the importance of employing only such material for work on the testis, since this organ is very sensitive to the general condition of the animal. It would appear, therefore, that the time relations of the chief phases of spermatogenesis in the immature rat and mouse are remarkably similar and that in both mature spermatozoa are produced on or about the 40th day.

Allen points out that according to Donaldson's tables (1924) the testes of the rat weigh 0.067 gm. at 14 days old and 0.244 gm. at 37 days. Thus in the rat, the testes almost quadruple their weight from the time when pachytene nuclei are plentiful until the first crop of spermatozoa are produced. During the corresponding period in the mouse the weight of the testes increases from 12 ± 4 mg. at 14 days to 120 ± 20 mg. at 42 days, *i.e.*, by about 10 times their initial weight.

The male rat, according to Donaldson (1924), has a body weight of 38 gm. at 37 days and 280 gm. at 365 days when the curve is almost flat. Thus it may reach a body weight eight times as great as that when it first produced mature spermatozoa in the testis. The cleaned-body weight of the mouse is 13.5 ± 2 gm. at 42 days and 27 ± 5 gm. approximately at 300 days when the curve is flattening. Thus the mouse only doubles its weight subsequently to the appearance of mature spermatozoa in the testis. Taking the appearance

of mature spermatozoa in the testis as a satisfactory criterion of puberty it is remarkable that whereas the mouse only doubles its weight subsequently, the rat can attain a weight eight times that at puberty. The mean weight at birth of a male rat is 4.7 gm., according to Donaldson, while in the male mouse it is 1.41 gm., as given by Parkes (1926) using the same strain of mice, for the mean birth weight of males and females combined, a weight which conforms well with our data. Robertson and Ray (1916) record a birth weight in 56 mice of 1.23 gm., which is rather lower, and Kopec (1930) records a birth weight for male mice of 1.25 gm. It is apparent that the male rat attains a weight rather over eight times and the male mouse, taking Parkes' figure of 1.41 gm., rather over nine times its birth weight at puberty as judged by the presence of mature sperms in the testis. It is subsequent to puberty therefore that the notable difference in the growth of these two nearly related species occurs.

Reviewing the more salient points in the time relations of growth of the testis it is significant that the greatest increase in the diameter of the spermatic tubules, occurs at about a fortnight old, simultaneously with the appearance of the maximum number of spermatocyte nuclei in pachynema and of lumina in the tubules. The most rapid growth of the testes is taking place at 30 to 40 days, at the same time as the spermatic tubules constitute a maximum part of the testis, as shown by the relative area of intertubular to tubular tissue on age, fig. 9, and shortly before mature spermatozoa are produced. Finally the percentage body weight of the testes reaches a maximum at about 40 days at the time when the increase in the diameter of the spermatic tubules is ceasing, fig. 6, and when mature spermatozoa are produced, and the histological picture presented by the testis is that of a mature animal.

Summary.

(1) Approximately 140 male mice, all taken from an in-bred colony, whose ages ranged from birth to upwards of 300 days, were employed in this research.

(2) The cleaned-body weight and the weight of the testes was obtained for each animal.

(3) The data for the growth on age of (a) cleaned-body weight, (b) weight of testes, (c) mean diameter of spermatic tubules, (d) area of spermatic tubules in transverse section, (e) area of intertubular tissue in transverse section, together with the growth of testes on cleaned-body weight, are given in the form of scatter diagrams. A linear regression has been fitted for the right

testis on the left and it is shown that the right testis is significantly heavier than the left.

(4) The maximum number of primary spermatocyte nuclei in pachynema occurs on the 14th day, together with the appearance of a lumen in many of the spermatic cords, while at about the same time the increase in the mean diameter of the spermatic tubules attains a maximum and all the cords become luminate.

(5) Mature sperms appear first in the testis of the mouse on the 42nd day while in the rat they appear on the 37th day (Allen, 1918). The appearance of mature sperms in the testis provides an easily determined and clearly defined point in the period of puberty. From birth to puberty, so defined, the rat increases its body weight by eight times, the mouse by nine times. Afterwards whereas the rat attains a weight eight times that at puberty when it is a year old, the mouse only doubles its weight from puberty to about 300 days.

(6) The testes attain their maximum growth rate at 30 to 40 days, at the time when the area of spermatic tubules in a transverse section relative to the area of intertubular tissue is at a maximum.

(7) Although there is a greater tendency towards degeneration of a small percentage of germ-cells in some of the spermatic tubules, about the time when the majority of spermatocytes are in pachynema, no generalised wave of degeneration, such as has been described in some mammals, occurs between birth and maturity.

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The Elasticity of the Vitreous Body.

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In a series of previous papers (Duke-Elder, 1929) evidence has been presented that the vitreous body of the eye is an elastic protein gel in a state of high turgescence. About the physical nature of the system and the conditions which determine its stability little is known; and since these questions, in addition to being of theoretical interest from the standpoint of very dilute gels, are also of considerable moment from the practical point of view in the normal and pathological metabolism of the eye, they would seem to merit further consideration.

The very small rigidity of the vitreous body, and the ease with which its structure is broken down by mechanical stresses, make ordinary methods inapplicable to the study of its elastic properties. The only technique which appeared practicable was that of Freundlich and Seifriz (1923, 1924). These workers made determinations of the elasticities of a number of sols and gels by introducing a small particle of nickel into the system by a micromanipulator and needle, and attracting it by means of a suitable electromagnet; the particle was observed with a microscope, and the reversible displacements measured by means of a micrometer eye-piece. These methods were adopted with the introduction of some modifications rendered necessary in view of the peculiar nature of the vitreous body.

A small mass of the substance was introduced into a glass cell made from a piece of glass tubing, 6 mm. long and 10 mm. in diameter, ground square at the ends and cemented to a microscope slide with de Khotinsky cement. It could be closed on the top by means of a cover slip, and made air-tight with vaseline to prevent contamination or evaporation. This was clipped to the stage of a standard Leitz microscope, fitted with two objectives—No. 1*, 42 mm., and No. 3, 16 mm. The microscope was fitted with a Leitz micrometer eye-piece, in which the cross-hair was moved by a screw, bearing a drum divided into 100 divisions. Calibration with a Zeiss object-micrometer showed that, using objective No. 3 with the draw-tube right in, one division on the drum was equivalent to $0.472 \pm 0.001 \mu$. The micromanipulator was of the

ordinary pattern, made to clamp on to the stage of the microscope; three screws gave movements in three directions at right angles, and several coarse adjustments were also provided. The needles for use in the micromanipulator were drawn from 3 mm. Jena glass rods; with the use of a microburner, followed by a hypodermic needle (No. 14) as burner, needles of 10–20 μ could readily be made and bent at right angles near the point. Such a needle was fitted with plasticine into the holder of the micromanipulator and a nickel particle affixed to its point in the following manner.

Some of Kahlbaum's pure nickel powder was placed on a microscope slide and distributed by tapping, the excess being shaken off. The slide was placed on the microscope stage, and examined with the No. 3 objective, a particle about 50 μ in diameter and as nearly spherical as possible being brought to the centre of the field. The needle in the micromanipulator was then dipped in glycerine and slowly withdrawn. (If the needle is withdrawn too quickly, drops form on it instead of a uniform film.) Thereafter the manipulator was clamped to the stage and, using objective No. 1*, the point of the needle was brought fairly close to the particle selected. Using objective No. 3, the needle was lowered so that its point touched the top of the particle, and then raised, when the particle usually adhered to the point. The magnet consisted of an iron core, 54 cm. long and 25 mm. in diameter, on which was wound a coil of No. 24 S.W.G. enamelled copper wire, to a resistance of 280 ω . The coil was connected, through a rheostat, ammeter, and switch, to the 200 volt D.C. mains. To the end of the magnet core was screwed a horizontal iron rod, 5 cm. long and 6 mm. in diameter.

The eyes of cats and oxen were normally employed, and, especially in those cases when the vitreous humour was kept over any length of time, in order to preclude putrefactive changes minute precautions were taken to maintain sterility both of the vitreous itself and all the instruments and apparatus which came into contact with it. When due precautions were taken it was found that vitreous could be kept in the cell for periods up to 10 days without undergoing any appreciable change. The eye was excised and freed from adhering tissues and, after an area in the equatorial region had been seared with a hot iron, an incision was made and a portion of vitreous squeezed out into a sterile dish. A portion of this was then removed by forceps and placed in the cell upon the microscope stage.

In the introduction of the nickel particle into the vitreous gel a departure had to be made from the technique of Freundlich and Seifriz. If the needle with the particle were merely immersed in the gel it was found that the particle

did not normally come away from the needle in the gel, and, moreover, that the withdrawal of the needle caused a considerable disturbance, often lifting the mass of vitreous bodily out of the cell. The micromanipulator was therefore employed merely to place the particle on the surface of the gel. The cell was then closed with a cover-glass and placed on the top of the field magnet of a string galvanometer, so that its centre was just over the gap between the poles. The current was switched on, and, after about an hour, the particle was found to have penetrated 1 or 2 mm. below the surface.

After the particle had been introduced it was necessary to wait for an hour or two for the system to attain equilibrium. The cell was then placed on the microscope stage and arranged so that one edge of the particle was seen to coincide with the zero mark of the micrometer eyepiece (using objective No. 3). and measurements were made of the displacements produced on exciting the magnet.

When the end of the magnet was brought near to the cell and the current switched on, the particle could be seen to move, and to spring back towards its original position on switching off, thus showing that the gel possesses real elasticity. On the other hand, bringing the unexcited magnet near the cell produced no perceptible effect, showing that the residual magnetism of the core could be neglected. It was found, however, that, if the current was allowed to flow only for a very short time (*e.g.*, $1/5$ second), displacements could be obtained which were completely and instantaneously reversible, but that, if the current were left on for any appreciably longer time, the return of the particle was, in general, incomplete. Both the reversible and the irreversible components of the displacements were found to increase with the time for which the current was allowed to flow, a typical set of results being shown in Table I.

It is clear from these figures that the vitreous is not behaving like an ordinary

Table I.—Cat's Vitreous 2 Days Old.

	Time for which magnet was excited.	Reversible component.	Irreversible component.
		scale division	scale division
1	10 seconds	14.1	0.0
2	30 seconds	14.7	1.0
3	1 minute	17.4	1.6
4	3 minutes	23.1	6.0
5	9 minutes	35.0	16.3
6	10 seconds	26.6	1.4

elastic body, and it appears that its viscous properties must be taken into account. The fact that displacement No. 6 differs so greatly from No. 1 shows also that the behaviour of the gel is dependent on its previous history.

Freundlich and Seifriz appeared able to obtain for gelatine reversible displacements whose magnitude was independent of time, but the different behaviour of the vitreous humour is doubtless due to the very small concentration* of the gelling component. It is probable also that the micellar structure of the vitreous body, as is revealed by ultra-microscopic examination, may have a bearing on the question.

A consideration of the work of H. J. Poole (1925) on fairly concentrated (*e.g.*, 16 to 33 per cent.) gelatine jellies seems germane. Investigating the linear extension of cylinders of gelatine gels, produced by the attachment of weights, he found the extension to be dependent upon the time of straining, and plotted a number of time-extension curves for various loads. If E is the extension produced in time T , he found that the derived curve of dE/dT against E was straight over a considerable portion of its length, but later underwent a sudden inflection, the remainder being curved. On the basis of a mechanical analogy, he arrived at the conclusion that the stretching of a gel system consisting of a network of elastic fibrils suspended in a viscous liquid should be made up of two components—an instantaneous extension due to the sudden stretching of the fibrils, followed by a slower “creep,” during which the intermicellar liquid readjusts its position by viscous flow. Both these extensions should, of course, be reversible.

Poole deduced that this “viscous-elastic creep” should be in accordance with the equation

$$K_1E + K_2\eta (\partial E/\partial T) = \text{constant}, \quad (1)$$

where

K_1 is a constant proportional to the elasticity of the fibrils,

K_2 is a factor depending on the nature of the channel through which flow can occur,

η is the coefficient of viscosity of the intermicellar liquid,

E is the total extension, and

T is the time taken to produce this extension.

Transposing (1) and differentiating with respect to E ,

$$\frac{d(\partial E/\partial T)}{\partial E} = -\frac{K_1}{K_2\eta} \quad (2)$$

* Only 0.025 per cent.

In other words, the curve representing equation (1), with E and $\partial E/\partial T$ as variables, should be a straight line, the slope of which is inversely proportional to the viscosity of the liquid and to a factor depending on the nature of the flow-path, while being directly proportional to the modulus of elasticity of the fibrillæ. Since neither the dispersion nor the viscosity should be affected by variation of the load, the slope of the curve of equation (1) should be independent of the load. Poole's results upheld this explanation, and constitute strong evidence in favour of the micellar theory of gelatine gels. He found that the extension corresponding to the point of inflection of the $\partial E/\partial T$ — E curve represented the maximum reversible extension of the gel, and regarded the remainder of the extension as an irreversible deformation of the fibrillæ.

In order to investigate the motion of the particle in the vitreous humour from this standpoint, arrangements were made for observing the position of the particle at known intervals of time after switching on the magnet, to increase the accuracy of which, especially in the rapidly changing early stages, a metronome was used as time-recorder. A typical example of the curves so obtained with cat's vitreous is shown in fig. 1. It will be seen that the curve

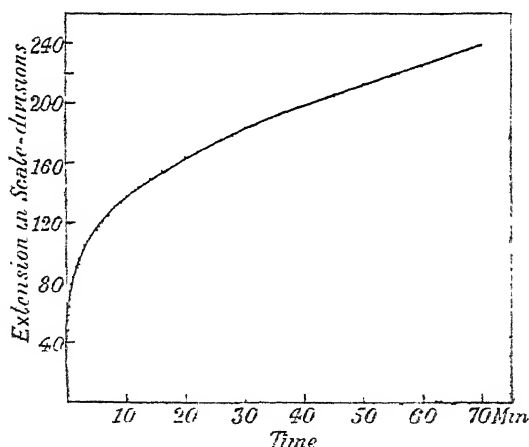


FIG. 1.

is initially very steep, but soon bends over, the final position being practically straight, corresponding to a uniform motion of the particle. while fig. 2 shows the derived curve, extension E being plotted horizontally and $\partial E/\partial T$ vertically. It will be seen that the earlier points (with the exception of the first) all lie close to a straight line, and that, at an extension of 81.3 divisions, there is a well-marked point of inflection, the curve thereafter tending to become asymptotic to the E -axis.

The general form of these curves is very similar to that of Poole's curves for gelatine, the discrepancy shown by the first point being doubtless due to the fact that the system had not settled down to a steady state after the first

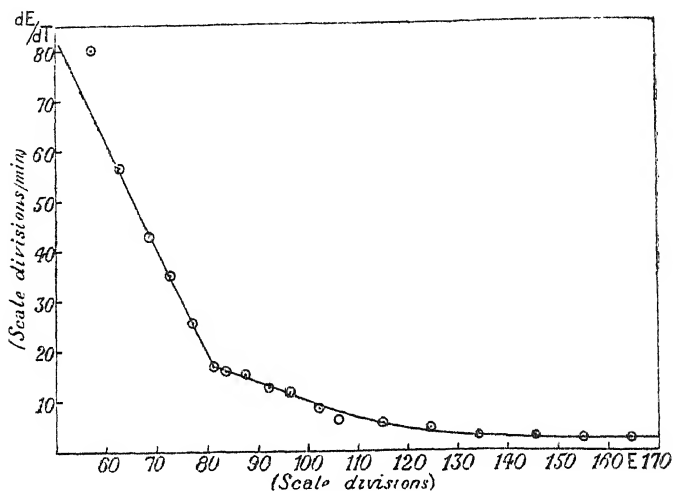


FIG. 2.

instantaneous displacement. This discrepancy of the first one or two points was almost always found to be present. The fact that the slope of the straight portion of the $\partial E/\partial T - E$ curve is independent of the force acting on the

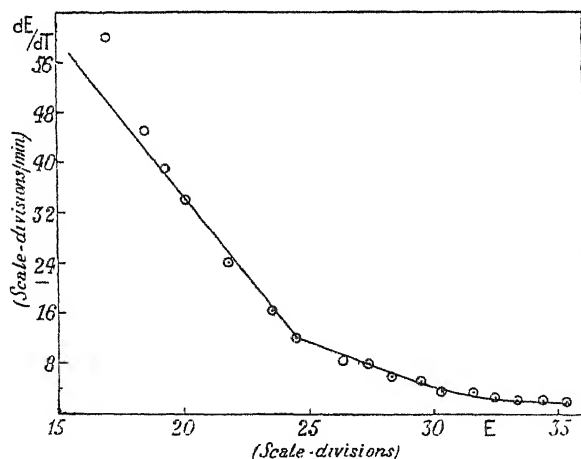


FIG. 3.

particle was shown by carrying out successive experiments with the magnet at different distances from the particle. In fig. 3 is shown the $\partial E/\partial T - E$

curve obtained with the end of the magnet at a distance of 12 mm. from the nickel particle, and fig. 4 shows the corresponding curve obtained when this distance was reduced to 8 mm. Neglecting the first two points, the slopes of the straight portions of two curves are found to be the same within experimental error, the value of $\partial^2 E / \partial T^2$ being 5.1 minutes.

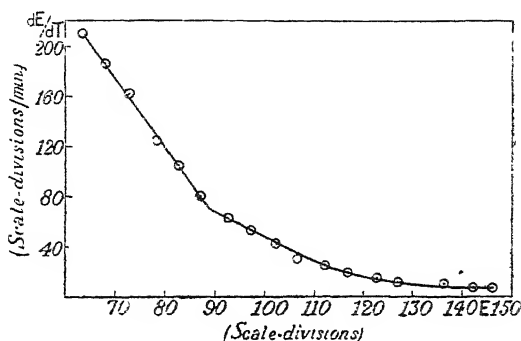


FIG. 4.

Up to this point, therefore, the displacement of the nickel particle in the cat's vitreous appears to follow the same laws as the linear extension of Poole's gelatine cylinders. Poole also found that the extension corresponding to the point of inflection of the $\partial E / \partial T - E$ curve was equal to the greatest reversible extension for the load in question, and was directly proportional to that load. The rate of return of the particle in the vitreous, after switching off the magnet, is so slow that the determination of its final equilibrium position is rather uncertain without careful control of temperature, etc. In the two cases, illustrated in figs. 3 and 4, the reversible components of the displacements appeared to be 38.2 and 100.8 scale divisions respectively, while the extensions corresponding to the points of inflection of the $\partial E / \partial T - E$ curves are considerably smaller, viz., 24.5 and 88.8 scale divisions. If we make Freundlich's assumption that the force acting on the particle is inversely proportional to the square of its distance v from the end of the magnet, then the displacement Δ corresponding to the point of inflection should be proportional to $1/N^2$.* The observed values do not bear out this relationship, being more nearly explained by the assumption of an inverse cube law, but there is little justification for either assumption, especially in view of the disturbances due to the steel microscope stand.

Vitreous humour from ox eyes appears to have much the same elastic

* By analogy with gelatine.

properties as that obtained from cats, although it is definitely less rigid, and gives curves of a slightly different shape. Fig. 5A shows the time-extension curve obtained with ox vitreous, the end of the magnet being at a distance of 15 mm. from the particle. Comparison with fig. 1 (cat's vitreous, 8 mm.) will show that, although in the case of the ox vitreous the field was weaker, the displacement produced was much greater, while the curve in fig. 5A bends over considerably more slowly than that in fig. 1. The size of nickel particle was approximately the same in both cases, and the current flowing in the magnet

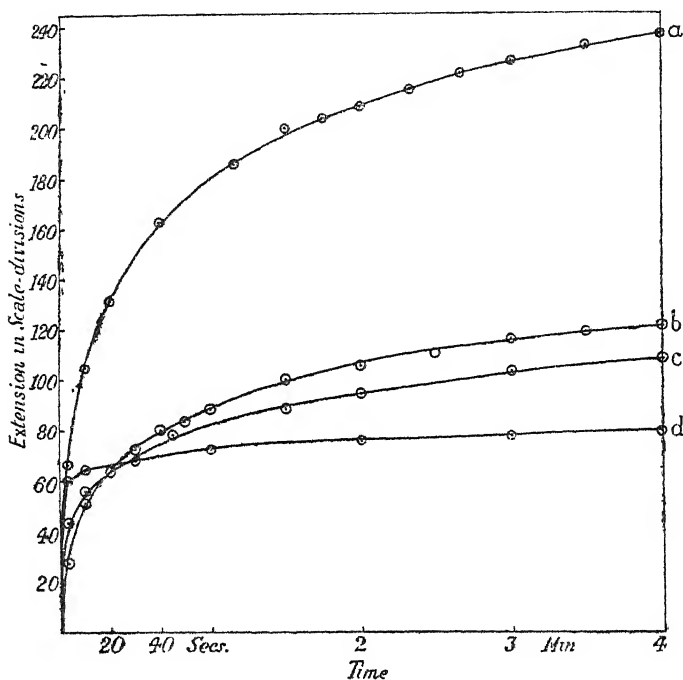


FIG. 5.

was identical, so that the experiments are quite comparable even if not quantitatively the same. The differences exhibited appear to be inherent ones, as the respective curves are not isolated examples, but typical of many experiments with different specimens. Fig. 6A shows the dE/dT curve derived from fig. 5A, the slope of the straight portion is 6.1 minutes. Figs. 5A and 6A show the corresponding curves for $v = 20$ mm.; in this case the straight portion of the derived curve had a slope of 6.8 minutes—the same within experimental error.

Acidification of the vitreous produces a considerable change in the shape of the T — E curve. Fig. 5C shows a curve obtained with ordinary ox vitreous

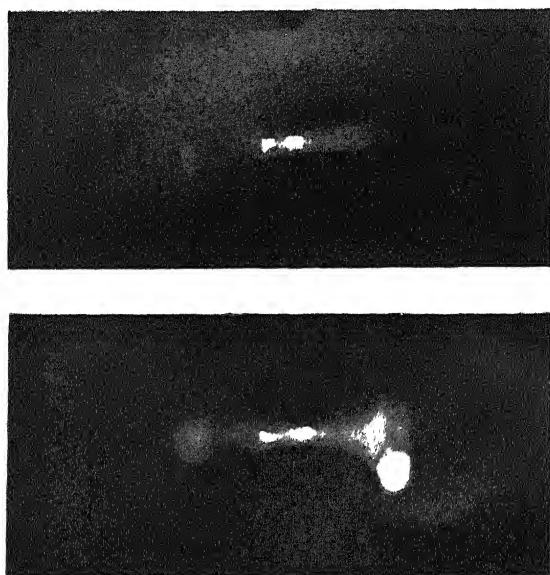


FIG. 1, *a* (above), *b* (below).—Ultra microscopic appearance of vitreous humour.

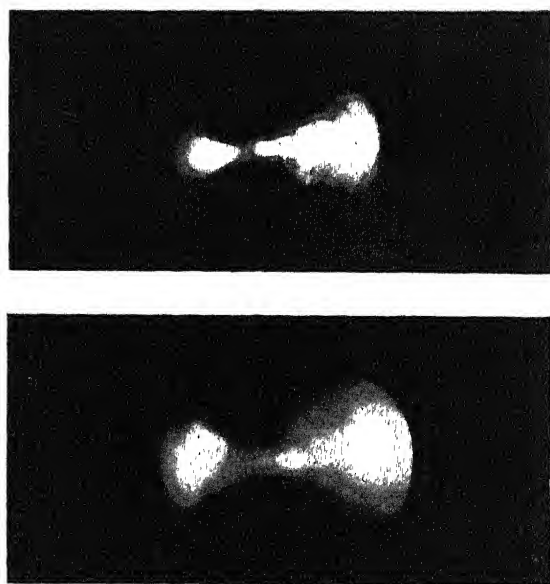


FIG. 2, *a* (above), *b* (below).—Ultra microscopic appearance of 1 in 50 plasma gel.

while in fig. 5D is shown the curve obtained after addition of a drop of very dilute hydrochloric acid, the other conditions remaining the same. It will be seen that the instantaneous displacement is much increased, while the "creep" is greatly diminished, so that the gel behaves more like an ordinary elastic solid. The reason for this effect is doubtless the shrinkage of the gel and withdrawal of water from the fibrillæ, although the acidification was not

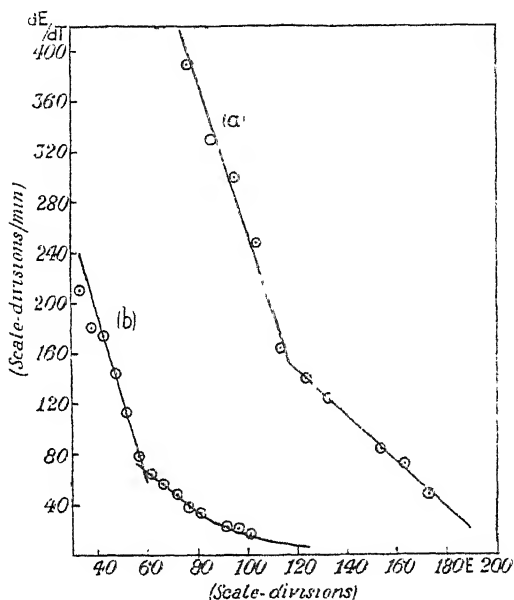


FIG. 6.

sufficient to modify the ultramicroscopic appearance of the gel appreciably. The effect of change of p_H and of various ions is one that would require careful study for its elucidation.

We have to acknowledge our indebtedness to the Medical Research Council for financial help, and to Professor F. G. Donnan for his personal help.

Summary.

(1) The nickel-particle technique of Freundlich and Seifriz has been suitably modified and applied to the study of the elastic properties of vitreous humour.

(2) The vitreous humour has been shown to possess viscous-elastic properties similar to those of gelatine.

(3) Ox vitreous is considerably less rigid than cat's, and gives time-extension curves which bend over more slowly.

(4) Acidification causes the gel to behave more like an ordinary elastic solid, increasing the instantaneous displacement and diminishing the "creep."

(5) The results indicate that the vitreous humour is a gel composed of a mesh-work of elastic fibrillæ suspended in a viscous liquid, a view which is borne out by ultramicroscopic examination.

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A Note on the Physical Properties of Dilute Plasma Gels and an Analogy with the Vitreous Body.

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[PLATE 5.]

A study of the physical properties of the vitreous body shows that it is a gel possessed of several characteristic physical properties (Duke-Elder, 1929), many of which seem to be dependent upon the great dilution of the gelable constituent (0.025 per cent.). It seemed of importance to determine whether these properties were unique or if they could be reproduced in another system. Observations were accordingly made of the behaviour of the gel which results on the coagulation of very diluted plasma.

Whole blood taken from a horse was transported to the laboratory in a cooled paraffin-coated jar to prevent coagulation, and was centrifuged in waxed tubes until a clear plasma resulted. This was diluted with normal saline in varying proportions and the resultant solutions allowed to coagulate at 30° C. It was found that the diluted plasma would not coagulate unless inoculated with a few drops of uncentrifuged blood, or some of the serum

expressed from a clot; thereafter, coagulation took place in 1 to 3 hours, the most dilute solutions being the last to clot.

The products obtained from the solutions whose concentrations ranged from 1 in 10 to 1 in 60 bore a striking resemblance to the vitreous body, especially that obtained from the solution of 1 volume of plasma in 50 volumes of normal saline. The concentration of fibrin in this gel is roughly the same as that of the residual protein in the vitreous body (viz., about 0.02 per cent.). The only material difference between the two systems was that the plasma gel was rather more rigid and friable than the vitreous body. Like the latter, the resultant gels were clear and almost colourless. When supported they were stable, but rapidly broke down under unbalanced mechanical stresses. Thus two experiments described in the case of the vitreous body can be exactly reproduced with diluted plasma gel. When the gel was suspended from a clamp, a clear liquid immediately began to drip away from it, until finally only a fine thread of fibrin was left adhering to the clamp. Similarly, when a portion of the gel was left on a filter paper, a clear liquid passed through, while a thin skin of fibrin remained behind.

Moreover, the ultramicroscopic appearances of the two are almost indistinguishable as is seen in figs. 1 and 2, Plate 5. The photographs were taken with an ultramicroscope of the immersion type by substituting a camera for the eye-piece of the microscope. In taking photographs some difficulty was encountered in the focussing, owing to the faintness of the image, and the slight Brownian movement tended to cause blurring, but some photographs were obtained which showed the fibrillar structure tolerably clearly. Wellington Ortho Process (Contrasty) plates, of speed 30 H. and D., were used, and developed with hydro-quinone and potash, the exposure being 10 to 15 seconds.

In order to make a more accurate comparison, the elastic properties of the plasma gel were investigated by means of the nickel particle method (Duke-Elder and Robertson, 1932). Some difficulty was encountered in the introduction of the particle, as a skin tended to form at the surface of the gel, which was drawn in by the particle and interfered with its subsequent movements. This difficulty could, however, be largely overcome by placing the diluted plasma in the cell of the microscope before coagulation, introducing the particle, and allowing the solution to clot with it in position. The gel from the 1 in 50 plasma solution was found to be much more rigid than even cat's vitreous humour, and a particle about 0.3 mm. in diameter had to be employed to give reasonable displacement.

The general elastic behaviour of the plasma gel was similar to that of the vitreous body. On starting the current in the magnet, the particle suffered

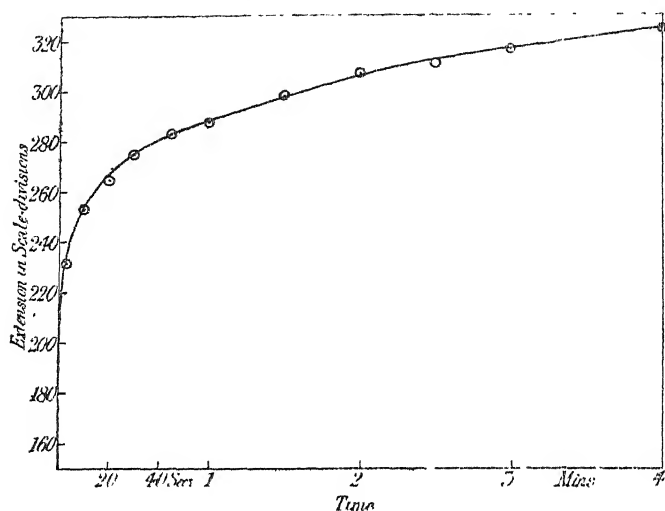


FIG. 3.

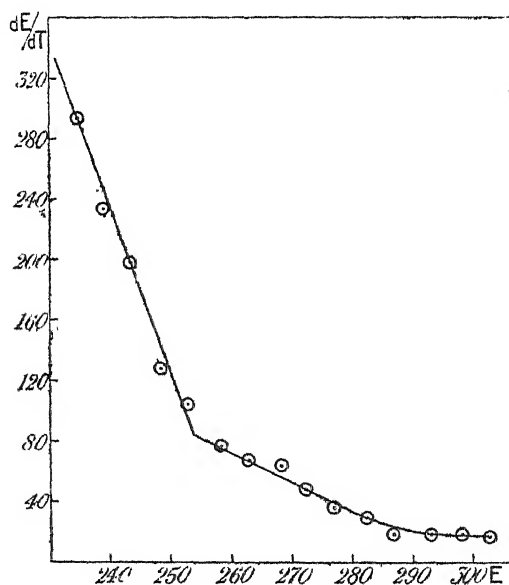


FIG. 4.

an instantaneous displacement, followed by a slow viscous-elastic creep. The instantaneous component of the displacement was, however, a much greater fraction of the whole than in the case of the vitreous body, and the rate of

creep diminished much more rapidly. This considerably increased the difficulty of observing the initial phases of the displacement.

The time-displacement curves were similar to those obtained with the vitreous body, but the $dE/dT - E$ curves were somewhat inconsistent. As a rule they were of the usual type—a straight initial portion, followed by a well-defined break, the curve then becoming asymptotic to the E axis. A typical pair of curves is shown in figs. 3 and 4. Sometimes, however, a more or less smooth curve was obtained, showing no obvious straight portion or point of inflection. These inconsistencies are due to the difficulties of observing the rapid initial phases of the displacement.

Conclusion.

The physical appearance and properties of the vitreous body are not unique, but can be to a large extent simulated by a dilute plasma gel.

We have to acknowledge our indebtedness to the Medical Research Council for financial help, and to Professor Drummond for his personal help.

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Comparative Studies on the Physiology of the Iris.—I. Selachians.

By JOHN Z. YOUNG, B.A.

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I. *Introduction.*

The iris of fish first attracted attention on account of the fact that its sphincter muscle contracts under the influence of light, even after the eye has been removed from the head. This phenomenon has been studied by Steinach, Magnus, Guth (1901), Franz (1905 and 1906), and recently by Herk (1928 and 1929). These researches have shown that in Cyclostomes, Selachians and many Teleosts the incidence of light gives a stimulus directly to the sphincter muscle (without the mediation of any nervous mechanism), causing it to contract. It has recently been shown, however (Young, 1931), that in one Teleost at least, *Uranoscopus*, there is a nervous mechanism and that the direct effect of light on the iris plays no important part in its normal movements. This mechanism itself is of considerable interest in that it is the reverse of that found in Mammals, and it seemed worth while to make a closer examination of the iris of other fish in order to discover whether a nervous mechanism is present in any other cases.

The pharmacology of the iris has never been thoroughly studied. Steinach, Beer (1894) and Guth (1901) showed conclusively that atropine, even in concentrations of 2 per cent., did not, at least for a considerable time, inhibit the narrowing caused by illumination of the pupil of an isolated eye. Young (1931) showed that pilocarpine, eserine, acetyl choline, and adrenaline all constrict the pupil of *Uranoscopus*, but the methods used were rough.

The aim of the present work has been to make a thorough comparative study of the iris of fish, in the hope not only of revealing the special and peculiar mechanisms present and their phylogenetic relationships, but also of contributing to the general physiology and pharmacology of smooth muscle, and to our knowledge of bio-photochemical reactions.

II. *Methods.*

Measurement of the diameter of the pupil of the living animal was made by means of fine screw dividers, the lengths being read off on a millimetre diagonal scale. The maximum error of this method is 0.2 mm. In order to

obtain more rapid and accurate measurements of the movements, the isolated eyes were examined by reflected light, under a microscope provided with a camera lucida. The eyes were pinned down in capsules whose bottom had been covered with paraffin wax, and illuminated from above by a focussed beam of light. Coloured filters enabled the effects of sudden illumination, etc., to be studied. In this way the diameter of the pupil can be recorded accurately on a piece of squared paper placed under the mirror of the camera lucida, or alternatively the movements of a single point at the margin of the pupil followed by making a prick on the paper at regular intervals of time, to correspond to the position of the pupil. The squared paper was illuminated by a separate lamp, shaded from the iris by a black screen and a stop watch was placed on the paper so as to fall in the same field of vision. For small pupils a $2/3''$ objective could be used, giving a magnification of $53\times$, allowing of measurements with a maximum error of 0.02 mm. For the larger pupils of Selachians a $1\frac{1}{2}''$ objective was used giving a magnification of $20\times$ and an error of 0.05 mm. The method has the great advantage that absolute measurements can be made, or movements followed, without imposing any strain on the muscle.

During the experiments the muscles were kept under a constant stream of oxygenated isotonic solution provided from a large vessel through which oxygen was continually bubbled, and delivered through a fine opening which was adjusted so that the stream of liquid did not disturb the iris. By means of a two-way cock different solutions could be run on to the muscle and afterwards washed away with a fresh stream of isotonic solution.

In the earlier experiments the eyes were simply placed in capsules containing a known quantity of isotonic solution, the drugs to be tested being added with a pipette to give the required concentration.

The various isotonic solutions used are described in a separate paper (Young, 1932, *a*). The p_H in all cases was kept between 7.3 and 7.6 . The temperature was measured before and after the experiments and did not vary by more than 0.5° C. during any one experiment.

All the drugs used were obtained from the British Drug Houses, Ltd. Solutions were made up immediately before use. Adrenaline was weighed out as the base and dissolved in an equivalent quantity of $N/10$ HCl.

III. Previous Work.

Franz (1905 and 1906) pointed out that the Selachians could be divided according to the structures of their eyes into day-feeding forms (*e.g.*, *Acanthias*),

night-feeding forms (*e.g.*, *Scyllium*, *Raja*) and deep-sea forms such as *Spinax*. In the first group the pupils are wide open in the day time, whereas in the second they close almost completely; in the third group the eyes are very large and have wide pupils with a very weak iris musculature. He examined the iris of a number of forms histologically and showed that there are well-developed circular sphincter and radial dilatator muscles, both muscles being unstriated, and the sphincter containing pigment.

In *Acanthias* he found that illumination of the isolated eye was followed by partial closure of the pupil, which then proceeded to re-open when left in the dark. Electrical stimulation of the iris, even with strong currents, produced no movements. He therefore concluded "mit grösster Reserve" that the iris of *Selachians* receives no motor nerves.

Herk (1928) made a few observations on the pupil of *Raja*, and found that the only effect of changes of illumination on the isolated iris was a slight constriction at transference from darkness to light and *vice versa*.

IV. *Scyllium*.

(i) *Innervation of the Iris*.—The members of this genus are nocturnal and the pupils remain closed when illuminated, even with a red light. The pupil is round when it is open, but the closed pupil is elongated antero-posteriorly, so that when it becomes very narrow the dorsal and ventral margins meet, leaving narrow apertures in front and behind. The closure on illumination takes place surprisingly slowly in the living animal; for instance, after illumination with a hand torch pupils were often observed to take upwards of 2 minutes to become fully closed. This is the first piece of evidence that the closure is the result of the direct effect of light on the sphincter muscle, without mediation of a nervous mechanism, since where the latter is present (as in *Uranoscopus* and Mammals) the movements are much more rapid.

The re-opening which takes place in the dark is also a slow process and the rate seems to depend on the intensity and duration of the previous illumination, but exact experiments to test this were not made.

In order to stimulate the cranial nerves the animals were either lightly anaesthetised with chlorotone, or decapitated, or else the spinal cord was destroyed with a wire. The nerves were stimulated with weak induced "faradic" currents applied by means of "bipolar" or "unipolar" electrodes. The diameter of the pupil was measured with a fine pair of screw dividers.

Stimulation of the oculomotor nerve of a dog-fish whose eyes were illuminated with diffuse daylight had no effect on the pupil, which remained minimal. If

the fish had previously been left in the dark, so that the pupils became wide, and the oculomotor nerves were then stimulated, there was usually no effect on the iris, but occasionally slight dilatation was observed. If now the eyes were both illuminated until the pupils just became minimal and were then replaced in a weak red light, then stimulation of the oculomotor nerve on one side caused this pupil to re-open much more rapidly than the control. However, after the stimulation had ceased the pupil returned to the same size as its fellow. Stimulation of the ciliary nerves had similar results, the dilatation being even more marked. The results of such an experiment are shown graphically in fig. 1. It seems that the oculomotor nerve contains motor fibres to the dilatator

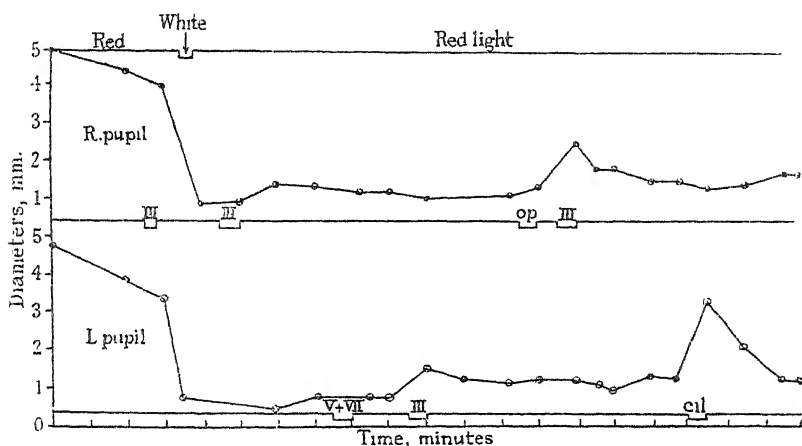


FIG. 1.—*Scyllium catulus*. Diameters of pupils. Spinal cord pithed. Measurements with dividers, maximum error 0.2 mm. Stimulation of nerves, with unipolar electrode and weak faradic currents, at the points shown. III, oculomotor nerve; op., ophthalmic branches of V and VII; V + VII, roots of trigeminal and facial; cil., anterior and posterior ciliary nerves.

muscle, but that the latter is very weak so that the effect of stimulating it does not appear when the tonus of the sphincter is high on account of illumination of the iris.

Stimulation of the other cranial nerves was without effect on the pupil, fig. 1.

The sympathetic system of the trunk sends no branches to the head of Selachians (Young, 1932, *b*).

After section of the oculomotor nerves or removal of the eyes from the head, the pupil became somewhat narrower and then closed on illumination much more rapidly than in the living animal; using a hand torch an exposure of 20 seconds instead of 2 minutes was sufficient to cause complete closure. This

confirms the hypothesis that the dilatator muscle receives motor fibres via the oculomotor, so that after section of this nerve (resulting in atonia of the muscle) constriction takes place more easily.

The course of the closure of the isolated eye of *Scyllium* in response to illumination was followed by means of the camera lucida method. As will be seen from fig. 2 the closure was at first rapid and then became slower; but not enough experiments have yet been made to allow any conclusions to be drawn as to the nature of the process involved.

When left in a white light the pupil of the isolated eye of *Scyllium* remained minimal indefinitely; there was no "adaptation" to the light, or fatigue of the mechanism responsible for the closing of the pupil. On transference to a weak light the pupil was seen to open, but only very slightly, fig. 2. Since presumably the photochemical processes which caused contraction of the

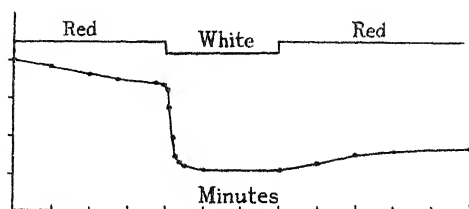


FIG. 2.—*Scyllium catulus*. Movements of dorsal margin of pupil of isolated eye in response to illumination. Cornea intact. Magnification 20 \times .

sphincter muscle are reversed in the dark, it must be assumed that the failure of the isolated iris to re-open is due to the absence of motor impulses to the dilatator muscle and to the weakness of the latter. This is confirmed by the fact that after stimulation of the nerve endings in the dilatator muscle with drugs, great dilatation can be obtained in the isolated eye (p. 233).

In order to test the effect of light on isolated pieces of sphincter and dilatator muscle, a strip was cut away round the inner margin of the iris. In this way all the sphincter tissue was removed and the dilatator left in place, the whole operation being performed in a weak red light. The strip containing the sphincter is difficult to deal with since it is very small and curls up, but on illumination it was seen to make very distinct movements, and when returned to the dark to return part of the way to its original position (*cf. Trygon*, p. 237). On the other hand, transference from red to white light or *vice versa* caused no movements of the dilatator muscle, although the action of drugs showed it to be capable of contraction.

The fact that the dilatator muscle receives motor fibres from the oculomotor

nerve was confirmed by sectioning this nerve intra-cranially under anaesthesia and allowing the animal to recover. The pupil on the operated side was then always seen to be narrower and to close more rapidly and open more slowly than the control (six experiments). For example, two days after section of the left oculomotor nerve (*S. canicula*, Naples, July), and after leaving the fish continually in the darkness, the diameter of the right pupil was 5.7 mm. and of the left pupil 2.5 mm.

After section of the ophthalmic branches of V and VII, including the profundus, the pupil was first seen to be narrower on the operated than on the control side when the fish was left in the dark, but within 24 hours this difference disappeared.

(ii) *Action of Drugs on the Iris*.—In these experiments the animal was left in the dark until the pupils became wide and was then decapitated and the eyes removed in a weak red light, pinned down in small capsules, and studied by the camera lucida method. After removal of the eye from the head the pupil always became somewhat narrower, probably on account of the removal of tonic influences to the dilatator muscle. After removal of the cornea there was further considerable narrowing; a similar narrowing is seen in mammals and is probably due to the fall of pressure resulting from escape of the aqueous humour.

1. *Eserine Sulphate*.—When applied to an illuminated (narrow) iris this drug had no apparent effect at any concentration up to 1 per cent., but if the eye was then removed to complete darkness the pupil slowly dilated (seven experiments). Solutions of 1/1,000,000 and 1/100,000 were without effect; 1/10,000 caused dilatation from 0.75 mm. to 1.43 mm. in 1 hour, whereas during the previous hour the pupil had narrowed from 0.85 to 0.75 mm. (maximum error 0.05 mm.). With stronger solutions greater dilatation was obtained; thus 1/3000 caused dilatation in 80 minutes from 0.15 to 4.00 mm., whereas the control, during the same time, opened only from 0.50 to 0.90 mm. (error 0.05 mm.). The process of opening was in all cases very slow and was inhibited by bright red as well as by white light. After treatment with eserine sulphate (even 1 per cent. for 1½ hours) the pupil was still able to close completely on illumination.

2. *Acetyl Choline Bromide and Choline Chloride*.—Solutions of acetyl choline bromide between 1/1,000,000 and 1/10,000 were all found to cause slow dilatation in the dark (11 experiments). For instance, a pupil opened in 1/10,000 solution from 0 to 3 mm. in 80 minutes and was then washed in isotonic solution in which it gradually narrowed again to 1.3 mm.; the control remained

minimal throughout. After the dilatation produced by acetyl choline the pupil closed readily on exposure to white light.

The addition of atropine inhibited the dilator effect of acetyl choline. For example, an iris placed in acetyl choline bromide 1/10,000 + atropine sulphate 1/100,000 narrowed during 2 hours from 0.87 mm. to 0.50 mm. whereas the control in acetyl choline bromide 1/10,000 dilated from 1.05 mm. to 4.90 mm.

In three experiments acetyl choline was tested on the isolated dilator muscle (prepared as on p. 232) and found to cause contraction, fig. 3.

Acetyl choline bromide injected subcutaneously caused mydriasis. After injection of 1 mg., for instance, the pupil was seen to dilate from 5.9 almost

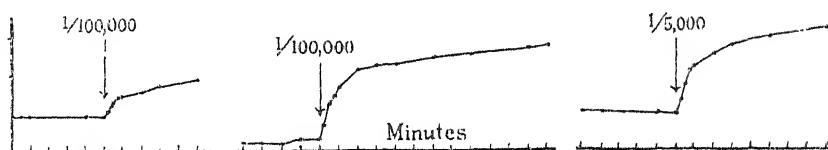


FIG. 3.—*Scyllium canicula*. Dilator muscle only. Measurements are of the diameter of the pupil after sphincter has been cut away. Current of oxygenated isotonic solution, p_H 7.6, temperature 27.3° C. Acetyl choline bromide added as indicated and washed off in between with isotonic solution. Magnification 20 \times .

immediately to 6.8 mm. and then to a maximum of 7.6 mm. 80 minutes after the injection. Six and a half hours later the diameter was still 7.3 mm. (maximum error 0.2 mm.)

In two experiments choline chloride 1/10,000 was found to be without effect on the pupil of the isolated dark-adapted eye, whereas 1/1,000 caused slight dilatation.

3. *Adrenaline Hydrochloride*. This seemed to have no very decided effect on the isolated iris of *Scyllium*. In three experiments in which it was added in concentrations between 1/10,000 and 1/200,000 to dark-adapted pupils no immediate effect was observed, and on illumination the pupils closed normally. Neither, in two experiments, did adrenaline hydrochloride 1/100,000 cause opening of the closed pupil in the dark, although subsequent addition of acetyl choline 1/10,000 did so. Addition of adrenaline hydrochloride 1/10,000 caused slight contraction of the isolated dilator muscle, but subsequent addition of acetylcholine bromide 1/10,000 caused very much greater contraction. Injection of adrenaline subcutaneously caused mydriasis, which was especially pronounced in eyes of which the oculomotor nerve had been cut. Thus three days after the operation (Naples, July) injection of 1 mg. caused dilatation of the pupil on the operated side from 3.0 to 6.0 mm. and of the control pupil

from 6.0 to 6.4 mm. The effect cannot, therefore, be central, but might be on the cells of the ciliary ganglion. However, in other Selachians adrenaline stimulated the dilatator muscle itself, and this is probably its action also in *Scyllium*; but since the effect is very weak, it was not observed on the pupils of the isolated eyes. Evidently, therefore, adrenaline has a stimulating action on the dilatator muscle, but this is less than that of acetyl choline and difficult to observe on the isolated iris.

4. *Pilocarpine Nitrate*.—This had no effect on the isolated, dark-adapted iris, even in solutions of 1 per cent. Subsequent illumination caused normal closure. The closed pupil was caused to re-open, in the dark, by the addition of pilocarpine, and this effect was inhibited by atropine. For instance, pilocarpine nitrate 1/10,000 caused dilatation from 0 to 2.3 mm. in 40 minutes, the control opening only from 0 to 0.25 mm. Atropine sulphate was then added to give a concentration of 1/10,000 which resulted in a slight narrowing (to 2.05 mm.); illumination for 2 minutes then caused closure to minimal, and during the subsequent 45 minutes in the dark there was re-opening only to 0.20 mm. (maximum error 0.05 mm.).

5. *Atropine Sulphate*.—Solutions of 1/100,000 to 1/100 had no immediate apparent effect on light- or dark-adapted pupils, nor did they inhibit the closure caused by the incidence of light. Guth (1902) found that even hours after the application of 2 per cent. atropine there was still normal closure of the pupil of the eel. As stated above, atropine was found to inhibit the dilatation caused by acetyl choline and pilocarpine. Injection of atropine sulphate subcutaneously caused *miosis*; for instance, 1 mg. caused the pupil to narrow from 2.0 to 1.6 mm., but the effect lasted only for about 1 hour; presumably it is due to the paralysis of the motor endings of the fibres in the dilatator muscle. Beer (1890) did not notice any changes in the pupil of *Scyllium* after injection of atropine.

V. *Mustelus*.

Mustelus, like the *Acanthias* (*Squalus*) investigated by Franz (1906), is a shark which hunts by day. The pupil is almost round and in ordinary daylight can be seen to be wide open. It would be interesting to discover what is the difference between the mechanisms for the regulation of the pupil of day- and night-feeding sharks. Since only a few *Mustelus* (*laevis* and *vulgaris*) were available, only a beginning could be made with this research.

In the living animal increase of the intensity of illumination of the eye caused narrowing of the pupil, which retained its round form, however, and

never became completely closed as does that of *Scyllium*. After a long stay in the darkness the iris became more sensitive, so that there was some narrowing on exposure even to red light.

When a *Mustelus* was suddenly illuminated and then left in the light, the pupils first became narrow, but then re-expanded, fig. 4, eventually returning after a long time to the original diameter. In *Mustelus*, therefore, unlike

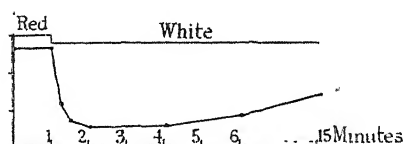


FIG. 4.—*Mustelus levis*. Variations in diameter of pupil of living animal in response to illumination. Measurements with dividers, maximum error 0.2 mm.

Scyllium, there is a possibility of adaptation, but this takes place much more slowly than in those animals which are known to have a fully innervated musculature (*Uranoscopus* and *Tetrapods*).

A similar adaptation was also seen in the isolated eye, figs. 5 and 6, the pupil in some cases re-expanding almost to its original diameter, while still in the

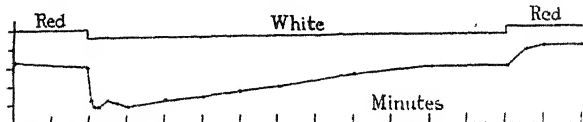


FIG. 5.—*Mustelus levis*. Movements of dorsal margin of pupil of isolated iris in isotonic solution, p_H 7.4; temperature 25.0° C. Magnification $53\times$.

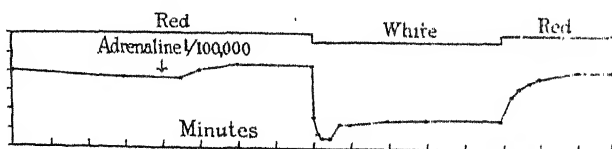


FIG. 6.—*Mustelus levis*. Movements of dorsal margin of pupil of isolated iris in isotonic solution, p_H 7.4; temperature 25.0° C. Adrenaline added to give concentration indicated. Magnification $53\times$.

white light, and always giving further sharp dilatation when transferred back to red light; so that in several cases it eventually became wider than before illumination. The process involved in the adaptation must be a reversal of the photochemical change responsible for constriction, since the dilatator muscle alone, after removal of the sphincter muscle, was found to make no movements on transference from red to white light or *vice versa*.

"Faradic" stimulation of the oculomotor nerve was not seen to cause any change in the diameter of the pupil in diffuse daylight (two experiments), but direct evidence that the dilatator muscle is stronger than that of *Scyllium* was obtained by the application of adrenaline, which immediately caused some dilatation, fig. 7, whereas in *Scyllium* it did so only very slowly if at all. After addition of adrenaline the effect of illumination was unaltered.

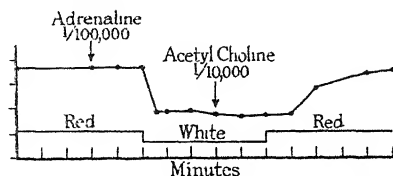


FIG. 7.—*Trygon violaceus*. Movements of edge of a piece of isolated sphincter muscle in isotonic solution, p_H 7.5; temperature 26.4° C. Magnification $20\times$.

VI. *Trygon*.

Trygon violacea is another day-feeding Selachian. In the aquarium it was seen to swim about during the day with the pupils dilated. As in other Rajiformes there is an "operculum iridis" present in the form of a flap which hangs down into the pupil from the dorsal margin of the iris.

Exact measurements of the diameter of the pupil of the living fish are difficult, since the iris, the outside skin and the retina are all of the same dark colour. No movements of the dark-adapted iris could be detected after stimulation of the oculomotor nerves.

After excision of the eye and removal of the back of the bulb and the cornea in a red light, it was found that illumination caused narrowing and often complete closure of the pupil, the operculum overlapping the ventral margin so as to leave only two narrow apertures in front and behind. As in the case of *Mustelus*, the pupil re-opened partially when left in white light—more so when returned to red light.

An isolated piece of sphincter muscle was seen to make movements when transferred from red to white light, fig. 7, and when returned to white light it returned to its previous position. Since there was no apparent tension on the muscle, it is difficult to see what can be the cause of this return.

Acetyl choline bromide caused immediate rapid dilatation of the pupil, fig. 8, and this indicates that the dilatator muscle of *Trygon* is stronger than that of *Scyllium*, since in the latter, acetyl choline only caused very gradual dilatation. Acetyl choline 1/10,000 had no detectable effect on the isolated sphincter muscle of *Trygon*, fig. 7.

Adrenaline HCl 1/100,000 caused in one experiment sharp initial constriction of the pupil, followed by dilatation, fig. 9. This was the only case in which application of any drug was seen to cause even temporary narrowing of

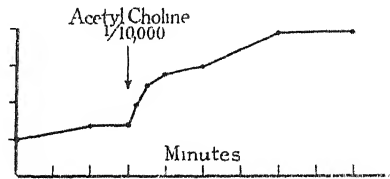


FIG. 8.—*Trygon violaceus*. Movements of ventral margin of pupil of isolated iris in isotonic solution, p_H 7.5; temperature 24.2° C. Acetylcholine added to give concentration indicated. Magnification $20\times$.

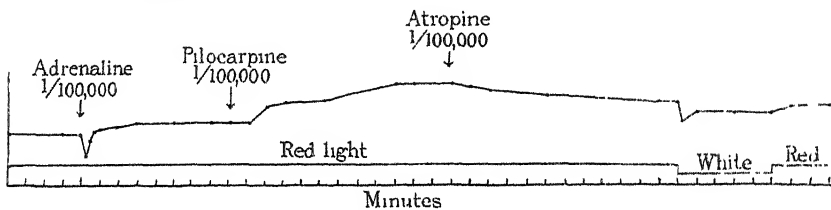


FIG. 9.—*Trygon violaceus*. Movements of ventral margin of pupil of isolated iris in isotonic solution, p_H 7.4; temperature 26.4° C. Drugs added to give the concentrations indicated. Magnification $20\times$.

the pupil of a Selachian. Probably it is to be explained as the result of an initial inhibition of the dilatator muscle, followed by stimulation. In other experiments adrenaline caused only dilatation, fig. 10, and further it was never seen to have any effect on the isolated sphincter muscle, fig. 7. The

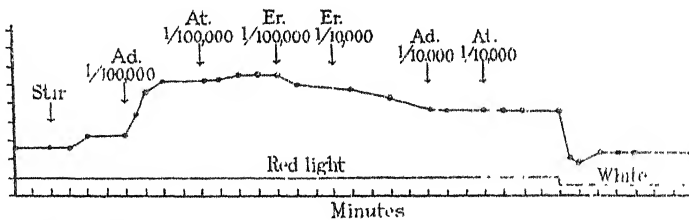


FIG. 10.—*Trygon violaceus*. Movements of ventral margin of pupil of isolated iris in isotonic solution, p_H 7.5; temperature 26.4° C. Drugs added successively to give the concentrations indicated. Magnification $20\times$. *Ad.*, adrenaline HCl; *At.*, atropine sulphate; *Er.*, ergotoxine phosphate.

dilatation produced by adrenaline was not inhibited by the addition of atropine sulphate 1/100,000, but on subsequent addition of ergotoxine phosphate 1/100,000 there was slight narrowing and more on addition of 1/10,000 of this drug, fig. 10. Further addition of adrenaline to give 1/10,000 was not

followed by any movements. After the addition of all these drugs, illumination still caused normal constriction followed by partial re-expansion, so that neither atropine nor ergotoxine affects the stimulation of the sphincter muscle by light.

Pilocarpine nitrate 1/100,000 caused further dilatation of the pupil after adrenaline dilatation and this effect was partly removed by the addition of atropine sulphate to make 1/100,000, fig. 9.

VII. *Discussion.*

There is evidence, then, that in the iris of all the Selachians studied the sphincter muscle is not under nervous control, but contracts in response to the stimulus of sudden increase in the intensity of illumination. The dilatator muscle, however, receives motor fibres from the oculomotor nerve. Franz (1906) was therefore partly correct in his surmise that the musculature of Selachians receives no motor nerves. In his most recent work (1931), published after the present researches had been finished, Franz was able to find nerve endings among the dilatator muscle cells, but not among the cells of the sphincter, thus bringing striking confirmation of the conclusions reached during the present work by quite other methods.*

In nocturnal sharks, such as *Scyllium*, the photochemical mechanism is so sensitive that the sphincter muscle contracts when exposed even to red light, and remains contracted for as long as it is illuminated. The dilatator muscle is very weak in these forms. In diurnal sharks such as *Mustelus* and *Trygon* the mechanism works on the same principle, but the photochemical mechanism reverses (or the sphincter muscle tires) during prolonged exposure to light, enabling the pupil to re-open. This process of adaptation is also helped by the fact that the dilatator muscle is stronger, relative to the sphincter in these diurnal sharks.

In the control of the pupil of Selachians there is thus an actual struggle or antagonism between the two muscles, since they are operated by two different, unco-ordinated mechanisms. In Mammals there is co-ordination, probably both centrally and peripherally, such that when one muscle contracts the other relaxes and the two work as synergists rather than as antagonists. The advantages of such a mechanism of excitation and inhibition over a primitive solution by conflict are seen when we compare the rapid movements and wide

* Bateson and later Franz (1931) noticed that in *Raja* there was contralateral closure when one eye was illuminated. If this is true it means that the mechanism in *Raja* differs from those described in the present paper.

possibilities of adaptation of the mammalian iris with the slow, stereotyped behaviour of that of Selachians.

The chief interest of the responses of the iris to drugs is that neither adrenaline, acetyl choline, pilocarpine nor eserine stimulate the sphincter muscle, nor do atropine or ergotoxine inhibit its closure in response to illumination. This is important evidence that these drugs act on the mioneural junctions in smooth muscle, a conclusion which is supported by the fact that all of the drugs have motor effects on the dilatator muscle which does receive nerves. The problem presented by the action of these drugs lies in the fact that although there is reason to suppose that their action is in some way linked with the mioneural junction, yet after degeneration of the nerves adrenaline and pilocarpine still exert their usual effects (Burn, 1922). "Das problem wäre leichter zu lösen, wenn es organe gäbe, zu denen keine Nerven, seien sie nun sympathisch oder parasympathisch hinführten" (Schilf, 1926, p. 68). The muscle required is provided by the sphincter iridis of Selachians, with a perfect control muscle close at hand in the shape of the innervated dilatator. Other nerveless muscles which have been investigated are the blood vessels of the placenta (Schmitt, 1922) and the amnion of the chick (Langley, 1905). In both cases some contraction was observed after addition of adrenaline, but the results were inconsistent. The present work seems to show that the effects of these drugs is connected with the mioneural junction and therefore that after degeneration of the nerve endings a muscle does not return to the condition of muscles which have never received nerves, but retains some part of the excitability caused by ingrowth of the nerves; "there was probably something at the nerve endings which did not degenerate when the nerve-endings degenerated" (Langley, 1921, p. 54).

Adrenaline and acetyl choline both have motor effects on the dilatator muscle, but the effect of the latter is the stronger. In this the dilatator differs from that of *Uranoscopus* where, although adrenaline sometimes causes dilatation, this is probably due to inhibition of the sphincter and not to stimulation of the dilatator.

As also in *Uranoscopus* it was found that atropine paralysed the motor action of pilocarpine and acetyl choline, but not that of adrenaline, whereas the latter was inhibited by ergotoxine, this being further evidence that the action of adrenaline is different from that of the other drugs in cases in which both groups act on the same muscle.

I am most grateful to Professor E. S. Goodrich for much advice and criticism and for reading the manuscript of this and the following paper.

VIII. Summary.

(1) The sphincter iridis muscle of *Scyllium*, *Mustelus* and *Trygon* contracts in direct response to illumination and is not under nervous control.

(2) The dilatator iridis of these forms receives motor fibres from the oculomotor nerve, after section of which the corresponding pupil remained narrower than its fellow. Stimulation of the oculomotor nerve was followed by dilatation of the pupil.

(3) Adrenaline, acetyl choline, pilocarpine and eserine all caused dilatation of the pupil and contraction of the isolated dilatator muscle. None of these drugs caused any movement of the isolated sphincter muscle.

(4) Atropine inhibited the dilatation due to pilocarpine and acetyl choline, but not that due to adrenaline; the effect of the latter was, however, inhibited by ergotoxine. Neither of these inhibitory drugs affected the response of the sphincter to illumination.

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Comparative Studies on the Physiology of the Iris.—II. *Uranoscopus*
and *Lophius*.

By JOHN Z. YOUNG, B.A.

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I. *Innervation of the Iris of Uranoscopus.*

The reasons for supposing that the sphincter iridis muscle of this fish receives motor fibres from the sympathetic system and the dilatator muscle motor fibres from the oculomotor nerve have already been stated (Young, 1931). In that paper certain rough pharmacological tests were reported, and these have since been supplemented by more exact observations with the camera lucida method which are here recounted.*

In the earlier work it was concluded since no further movements of the iris were observed after section of both optic nerves that the direct effect of light on the sphincter muscle plays no part in the control of the pupil of *Uranoscopus*. This has been confirmed with the more exact methods. Dark-adapted eyes were removed from the head in a weak red light, pinned down in a capsule and observed with a camera lucida. On illumination with a bright white light no movements were ever observed, although the magnification used ($53\times$) allows of the certain detection of movements of 0.02 mm. Since the diameter of the pupil of a medium sized *Uranoscopus* is about 2.3 mm., the smallest detectable movement would be equivalent to a narrowing of 1/115 of the diameter. Within this limit, therefore, increased intensity of illumination causes no movements of the isolated iris.

II. *Effect of Drugs on the Iris of Uranoscopus.*

These were tested by the methods already described (Young, 1932, *a*). The isotonic solutions used are described in a separate paper (1932, *a*). In all cases the cornea was removed and the whole of the back of the eye was cut away, in order to prevent disturbances due to movements of the retractor lentis muscle.

* The work on *Uranoscopus* was done at the British Association Table at Naples, for the use of which I am most grateful, as also to Professor Dohrn and everyone at the Station for their great kindness and help. The *Lophius* were obtained at Plymouth during a short stay at the Oxford Table, during which I received every assistance from Dr. Allen and his staff.

The degree of movement was not found to be the same all round the pupil. The point opposite to the operculum iridis showed the most vigorous movements and was therefore usually selected for observation. At the same time measurements of the diameter of the pupil were made, to allow of direct comparison of the effects of the drugs.

Pilocarpine nitrate (B.D.H.) was found to cause narrowing of the pupil in all cases (15 experiments). The greatest dilution at which an effect was observed was $1/10,000,000$ in which the pupil narrowed by $1/18$ of its initial diameter; in $1/100,000$ it narrowed by $1/1.7$ of the initial diameter, and in 1 per cent. it closed completely. As will be seen in fig. 1, the movement

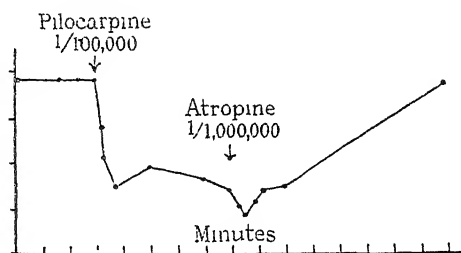


FIG. 1.—Movements of margin opposite operculum. Iris isolated in 20 c.c. of isotonic solution, p_H 7.3. Drugs added to give concentrations indicated. In all figures the time markings indicate minutes and the magnification is $53 \times$.

was very rapid and after the initial constriction the iris oscillated for a few seconds.

Arecoline Hydrobromide (B.D.H.).—In five experiments this drug was seen to cause constriction of the pupil, but this was less marked than that caused by pilocarpine. Thus $1/100,000$ caused narrowing by $1/2.7$ and $1/5000$ narrowing by $1/2.5$ of the original diameter. In one experiment $1/10,000$ arecoline hydrobromide, when applied after acetyl choline, was seen to cause

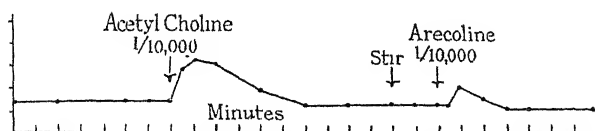


FIG. 2.—Movements of margin opposite operculum. Iris isolated in 20 c.c. of isotonic solution, p_H 7.4; T. 26.8°C .

a slight, but significant, initial dilatation which was followed by constriction, fig. 2; in three other cases tested it caused constriction after acetyl choline, fig. 3.

Eserine Sulphate (B.D.H.).—Solutions of $1/100,000$ of this drug caused no movements of the pupil (two experiments). $1/10,000$ caused constriction by

1/15 of the initial diameter, and 1/3000 by 1/8·6 of the initial diameter. After this constriction produced by eserine, pilocarpine nitrate 1/100,000 caused further narrowing.

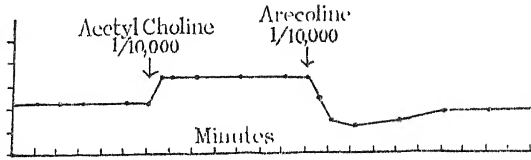


FIG. 3.—Movements of lateral margin. Iris isolated in 20 c.c. of isotonic solution, p_H 7·4; T. 26·0° C.

*Adrenaline Hydrochloride.**—In eight experiments, with dilutions varying from 1/1,000,000 to 1/10,000, this substance caused narrowing of the pupil, figs. 4, 5 and 6. With solutions weaker than 1/100,000 the effect was often absent. For instance, in one case 1/200,000 had no effect, further addition of adrenaline to make 1/50,000 caused constriction by 1/19, and to make 1/10,000 by 1/7 of the original diameter. The greatest narrowing observed was by 1/2, after addition of a solution of 1/10,000.

In two cases addition of adrenaline was followed by dilatation of the pupil. In the first, fig. 5, the solution used was 1/1,000,000 and the effect was twice repeated; stronger solutions then caused constriction. In the second case, fig. 6, the dilatation was temporary and was followed by constriction. These cases might be explained as due to stimulation of the dilator by the

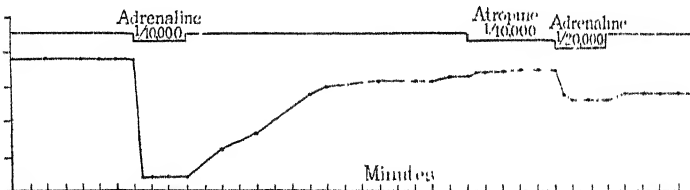


FIG. 4.—Movements of margin opposite operculum. Iris in continual stream of isotonic solution, interrupted as indicated. p_H 7·6; T. 25·8° C.

adrenaline, but an initial inhibition of the sphincter (especially with weak solutions) would be in accordance with the known effects of adrenaline on the blood vessels.

Acetyl Choline Bromide (B.D.H.).—The effects of this drug are complex and seem to differ with the concentration and with the state of the iris. Solutions weaker than 1/100,000 caused dilatation in seven experiments, fig. 8, but in one experiment 1/1,000,000 caused constriction, fig. 7. Solutions of

* Solutions all freshly made up by neutralisation of the base.

1/100,000 caused no movements in two cases, dilatation in three cases, and constriction in three cases. Solutions of 1/10,000 and stronger caused constriction in five and dilatation in two experiments. In experiments in which first weak and then strong solutions were applied, it was often seen that the former caused dilatation, and the latter constriction, intermediate concentrations having little or no effect. Thus in one case 1/500,000 caused dilatation by 1/13 of the original diameter, 1/100,000 caused dilatation by 1/20 of the

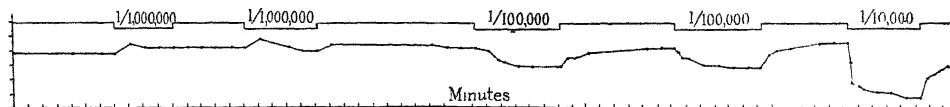


FIG. 5.—Movements of margin opposite operculum. Continual stream of isotonic solution alternating with adrenaline HCl at the concentrations indicated. p_H 7.7; T. 25.3° C.

original diameter, 1/5000 caused constriction by 1/6.7 of the original diameter. A similar phenomenon is seen in fig. 8.

The dilatations observed were too well-marked to be due to inhibitions of the sphincter (*cf.* the dilatations observed after adrenaline and ergotoxine which were of much less extent) and were probably due to stimulation of the dilatator muscle. Strong solutions, however, are evidently capable of stimulating the sphincter.

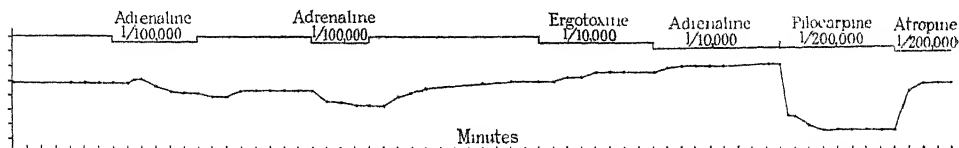


FIG. 6.—Margin opposite operculum. Continual stream of isotonic solution interrupted as indicated. p_H 7.4.

Ergotoxine Acid Phosphate (B.D.H.).—After application of this drug there were no immediate movements of the pupil, except perhaps a slight dilatation in two cases, but its effect was to inhibit the motor effect of adrenaline. Solutions more dilute than 1/100,000 were ineffective. After application of stronger solutions addition of adrenaline caused not constriction but slight *dilatation* of the pupil (three experiments). If the pupil was already constricted with adrenaline, and ergotoxine was then added, then there was slow dilatation, fig. 9. When adrenaline and ergotoxine were added together there was at first a constriction, but immediately the pupil began to re-open, and soon regained its previous diameter. After the addition of ergotoxine (1/10,000 or 1/5000) pilocarpine or concentrated (1/10,000) acetyl choline still caused

constriction. Neither does ergotoxine paralyse the motor endings of the sympathetic system in the sphincter muscle, as was proved by exposing the sympathetic chains of a freshly pithed animal, removing the corneas from the eyes and dropping ergotoxine phosphate 1/10,000 on to one of them. Five minutes later, stimulation of the sympathetic chain at the level of the vagus

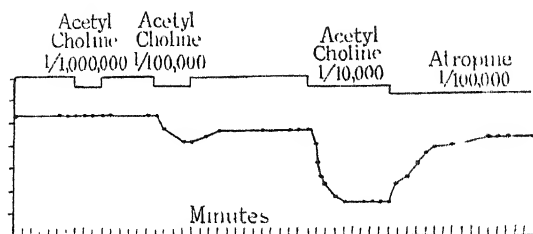


FIG. 7.—Margin opposite operculum. Continuous stream of isotonic solution interrupted as indicated. p_H 7.4; T. 25.4° C.

still caused as much constriction as before application of the ergotoxine. Atropine sulphate 1/10,000 was then dropped on to the eye and after 2 minutes stimulation of the sympathetic no longer caused any movements, even using very strong stimuli, although the control eye still reacted normally.

Atropine Sulphate (B.D.H.).—The action of this drug was to inhibit the motor effects of pilocarpine, acetyl choline or stimulation of the sympathetic nerves. Within less than 2 minutes of the application of a 1/1,000,000 solution all these stimuli were ineffective, and if atropine was added to

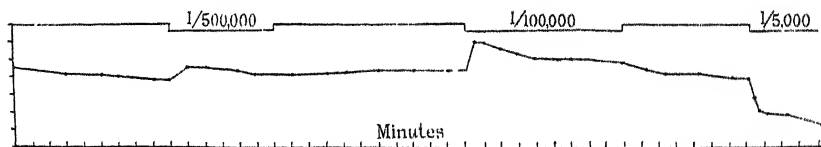


FIG. 8.—Lateral margin. Stream of isotonic solution alternating with acetyl choline bromide at the concentrations indicated. p_H 7.5; T. 27.1° C.

a pupil which was narrow under the influence of pilocarpine or acetyl choline, then there was immediate dilatation, figs. 1, 6 and 7. Atropine alone applied to the untreated iris caused no movements. The constrictor effect of adrenaline was not abolished by atropine 1/10,000, although it appeared to be somewhat less than before addition of atropine, fig. 4. In view of the fact that atropine almost instantaneously and quite completely inhibited the action of pilocarpine and acetyl choline, it is interesting that the action of adrenaline remains, even if impaired. Clearly the action of adrenaline on the neuro-muscular mechanism must be different from that of other drugs.

III. *The Pupil of Lophius piscatorius.*

This fish, like *Uranoscopus*, lives on the bottom of the sea and the eyes are directed upwards and used in the process of the capture of food. The iris resembles that of *Uranoscopus* in that it is thrown into radial folds (whereas

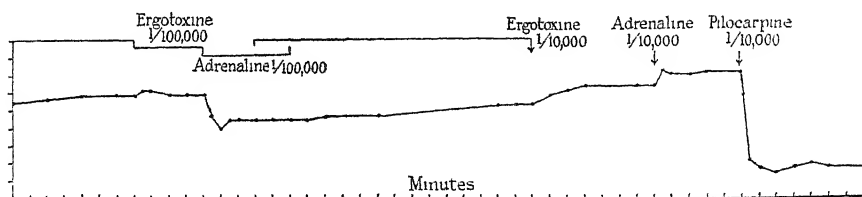


FIG. 9.—Margin opposite operculum. Stream of isotonic solution stopped before second addition of ergotoxine and thereafter drugs added direct and not in circulation. p_H 7.3; T. 24.5° C.

that of other Teleosts is flat and smooth) and in being capable of extensive and rapid movements.

The mechanism of the pupil was investigated in three specimens obtained at Plymouth. After "Faradic" stimulation, with weak currents, of the sympathetic chains behind the facial nerves* or behind the vagus there was constriction of the pupil, followed by re-opening after cessation of the stimulus. Stimulation of the short ciliary nerve had the same effect. No movements of the pupil could be detected after stimulation of the oculomotor nerve.

After section of one of the sympathetic chains in front of the vagus the corresponding pupil opened somewhat. An exactly comparable phenomenon was observed in *Uranoscopus* (Young, 1931), and is to be explained as due to release of the iris from tonic constrictor influences. After cutting the dorsal and ventral roots of the third and fourth spinal nerves close to the spinal column there was no change in the iris, but after cutting the second spinal roots there was some dilation, so that the constrictor fibres run in this nerve. In *Uranoscopus* the corresponding fibres run in the third and fourth ventral roots and in the frog Langley and Orbeli (1911) found dilatator fibres in the second and third spinal nerves.

In three experiments adrenaline 1/10,000 dissolved in 72 parts distilled water, 28 parts outside sea water, was found to cause considerable narrowing of the pupil.

Lophius is the only Teleost in which I have yet discovered an iris capable of rapid movements similar to those of *Uranoscopus*, and it is of interest that here

* The arrangement of the sympathetic supply to the head in *Lophius* appeared to be substantially the same as in *Uranoscopus*, but the nerves are much easier to find.

also there is a nervous mechanism involved, and that the synergists are arranged as in *Uranoscopus* and not as in Tetrapods. Taken together with the fact that in Selachians the dilatator muscle receives motor fibres from the oculomotor, this seems to show that the mechanism of fish in general is the opposite of that of Tetrapods.

IV. Discussion.

It seems, then, that pilocarpine, arecoline, eserine and adrenaline all stimulate the sphincter muscle of the pupil of *Uranoscopus*, while acetyl choline in weak solutions stimulates the dilatator and in stronger solutions also the sphincter. This is a curious complex of reactions. The effects of pilocarpine, arecoline and eserine are the same in *Uranoscopus* as in Mammals, but opposite to their effects in Selachians. It seems possible that these drugs stimulate the nerve endings both in the sphincter and dilatator muscles of *Uranoscopus*, but that since the sphincter muscle is much the stronger its action alone appears. This is confirmed by the fact that in one case arecoline caused dilation of the pupil. On the other hand, Poos (1927) was unable to find any effect of the parasympatho-mimetic drugs on the isolated dilatator of mammals.

The actions of adrenaline and acetyl choline in *Uranoscopus* are the reverse of those observed in mammals; that is to say, the effects of the drugs remain parallel to those of the nerves, adrenaline mimicking the effects of the sympathetic and acetyl choline those of the oculomotor. But the parallelism is not perfect, since acetyl choline in large concentrations stimulates the sympathetic endings in the sphincter.

The behaviour of adrenaline recalls its effects on the blood vessels of mammals, since it causes first slight inhibition and then contraction of the sphincter muscle, and ergotoxine prevents the stimulating action so that addition of adrenaline then only inhibits the muscle. In mammals small doses of adrenaline cause fall in blood pressure, as also after the action of ergotoxine. The question of whether this phenomenon indicates the presence of an inhibitory contribution from the sympathetic to these organs remains open (Dale, 1913).

It is of interest that atropine, which usually paralyses the action of the parasympathetic endings, here inhibits the action of the sympathetic endings in the muscles and also the motor action of pilocarpine and acetyl choline, but not that of adrenaline. Conversely ergotoxine, which inhibits the action of adrenaline, does not affect the response of the muscle to sympathetic stimulation (at least, in the doses used), nor the action of pilocarpine or acetyl choline.

Evidently the action of adrenaline on the muscle is fundamentally different from that of other drugs.

V. *Summary.*

- (1) Pilocarpine nitrate caused narrowing of the pupil, even in great dilution.
- (2) Arecoline hydrobromide caused narrowing of the pupil, but less than that observed after pilocarpine. In one case dilation was observed.
- (3) Eserine sulphate caused constriction in concentrations greater than 1/10,000, but the closure was only slight and pilocarpine caused further constriction.
- (4) Adrenaline HCl caused constriction; in two cases there was initial dilation, probably due to inhibition of the sphincter.
- (5) Weak solutions of acetyl choline bromide caused dilation, stronger solutions constriction of the pupil.
- (6) After the addition of ergotoxine phosphate, the effect of adrenaline was reversed so that it caused dilation of the pupil. Ergotoxine had no effect on the constriction caused by pilocarpine, acetyl choline or stimulation of the sympathetic nerves.
- (7) Atropine sulphate inhibited the constrictor action of pilocarpine, acetyl choline or sympathetic stimulation, but not that of adrenaline.
- (8) The iris of *Lophius piscatorius* resembles that of *Uranoscopus* in being capable of rapid movements under nervous control, the oculomotor dilating and the sympathetic constricting the pupil.

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Differences in the Growth of Transplantable Tumours in Plasma and Serum Culture Media.

By R. J. LUDFORD.

(From the Stroud Laboratory of the Imperial Cancer Research Fund.)

(Communicated by Dr. J. A. Murray, F.R.S.—Received November 1, 1932.)

[PLATE 6-9.]

1. *Introduction.*

A recent study of the behaviour of normal and malignant cells in tissue cultures of transplantable tumours (Ludford, 1932, *c*), carried out by means of vital staining has revealed a fundamental difference between them. When a plasma culture undergoes liquefaction the outgrowth of malignant cells collapses as the result of the breaking-down of the fibrin network by which the cells are supported. Malignant cells round off, usually become detached, degenerate and die. Non-malignant connective-tissue cells remain adherent to the cover-glass, and without renewal of the culture medium, often survive after all the malignant cells have died. Such behaviour suggests a difference between the plasma membrane of normal and malignant cells; the membrane of the malignant cell would appear to be less adhesive.

On the basis of these observations it appeared improbable that any outgrowth of malignant cells could occur in a serum medium. Since conclusions of far-reaching importance have been drawn from experiments with serum cultures of tumours, it seemed desirable to investigate this matter under controlled conditions.

2. *Technique.*

For our experiments we selected healthy areas of transplantable tumours of mice; these were cut into small fragments, and washed in Ringer's solution. Some were explanted in freshly prepared rat serum, others in rat plasma. Mouse serum and mouse plasma were also employed, with, and without, mouse embryo extract. There was no appreciable difference between growth in rat plasma and mouse plasma, or in rat serum and mouse serum. The method of Lumsden for adjusting the p_H of the serum has been employed. In most experiments rat serum and plasma have constituted the media, and one part of fowl plasma has been added to two parts of the plasma to retard liquefaction

of the clotted medium. Usually in each experiment 18 serum cultures, and 6 plasma cultures were set up. Since previous experiments had made us familiar with the type of growth in plasma, the smaller number of cultures in this medium was sufficient to show the state of activity of the tumour selected.

The tumours chosen for this work were the mammary carcinomata 63 and 206, and the Crocker sarcoma, and sarcoma 37 of this Laboratory. With each tumour three separate experiments were performed. Fluctuations in the rate of growth occur with transplantable tumours, so that by taking tumours of different generations of each strain it is possible to a certain extent to determine whether any differences in the behaviour of the cells in the two media is dependent upon their state of activity.

The cultures were vitally stained by adding a drop of 0.5 per cent. trypan blue (Grübler), in Ringer's solution as soon as growth was well marked, usually 24 hours after explanting. Plasma cultures were not sub-cultured, but the medium of the serum cultures was in most experiments pipetted off every day and fresh serum added. Vitally stained cultures at the same time received a fresh drop of trypan blue. In each experiment two or three cultures received no trypan blue. These served as a control to show whether the dye influenced growth.

Cultures were examined daily, and photographic records were kept of their mode of growth. Selected cultures were mounted in Ringer's solution, and examined with the oil-immersion lens, and after photographing were fixed in the "Susa" fluid of Heidenhain, and stained for 5 to 10 minutes in Rawitz carmine. These preparations constitute a permanent record of each experiment.

I have been assisted throughout this work by Mr. W. J. Dunn, the Tissue Culture Technician of this Laboratory, and our artist, Mr. A. V. Cobbett, has been responsible for the drawings and technical part of the photography.

3. *Methods of Differentiating between Malignant and Non-malignant Cells in Tissue Cultures.*

This analysis of the cells, which emerge from explants of transplantable tumours in plasma and serum, is based essentially upon the different reaction of normal and malignant cells to the acid dye trypan blue. It has been shown :

- (1) That *in vivo* malignant cells do not segregate trypan blue, while the connective-tissue cells of the stroma, and the cells of reaction tissue, monocytes, polyblasts, macrophages and fibroblasts do (Ludford, 1929).

- (2) That the same distinction between normal and malignant cells is retained *in vitro* (Ludford, 1932, b). Occasionally some dye droplets appear to be segregated in malignant cells, especially in those of the Jensen rat sarcoma. Large coloured bodies are also frequently found in the malignant cells of vitally stained tissue cultures; they are the result of phagocytosis.

Since objection has been raised against the employment of vital staining as a means of diagnosing specific cell types (Wallbach, 1931) this criticism has not been ignored in our work. Recent researches have emphasised that vital staining with acid dyes is to be considered as a physiological experiment, rather than a purely morphological technique. The state of activity of the cell determines the amount of segregation. Granular deposits of trypan blue are formed only by living cells. Dead cells stain diffusely by a passive process.

With the tumours used in our experiments, we have not found the malignant cells segregate trypan blue like non-malignant connective-tissue cells under any conditions of growth. On the other hand, normal healthy connective-tissue cells segregate dye *in vivo*, and in a similar manner *in vitro* in any medium which supports growth (Ludford, 1932, b).

We have occasionally had cultures to which trypan blue has been added in which there has been much less than the usual amount of segregation by connective-tissue cells. The reason for this has not always been clear. In some cases it has been due to a specially thick plasma clot; but there is the possibility that certain conditions may arise in a culture which retard the cellular mechanism of segregation.

Although vital staining with trypan blue has afforded the most striking method of distinguishing between normal and malignant cells, yet careful cytological examination enables most cells to be identified, once the differences have been seen in vitally stained cultures. Thus the malignant cells usually have larger nuclei, with relatively larger nucleoli. In carmine post-vitally stained preparations the cytoplasm of malignant cells is coloured more deeply than that of normal cells. Further in young healthy cultures the cytoplasm of malignant cells contains very few refractile granules, while the cytoplasm of cells of the polyblast type is full of vacuoles. These differences can be brought out more vividly by vital staining with a basic dye, such as neutral red. The cytoplasmic vacuoles are then stained intensely.

4. *Growth of the Transplantable Mammary Carcinoma 63 in Plasma and in Serum.*

The carcinoma 63 is a rapidly growing tumour, which rarely regresses *in vivo*. Its cells exhibit no indication of secretory activity. When explanted in plasma it invariably grows as a continuous sheet of cells, as shown in fig. 5, Plate 8. In cultures to which trypan blue has been added the cells comprising the sheet remain uncoloured, but other isolated cells, which extend far out into the medium, become filled with dye-droplets. The former are the malignant cells, the others, which segregate trypan blue, are cells of the mesenchyme-cell system—monocytes, polyblasts, macrophages and fibroblasts. On carefully focusing the microscope it is seen that the majority of these cells are adherent to the lower surface of the cover glass, while the sheet of malignant cells extends through the plasma clot. Fig. 1, Plate 6, is a drawing of a tongue-like outgrowth of carcinoma cells extending from the sheet of growth of a 3 days-old culture to which trypan blue was added on the second day. The isolated cells filled with granules are the vitally stained non-malignant cells. Sometimes the carcinoma cells grow in small isolated clusters, especially when the explant is largely necrotic. One of these islets of malignant cells (C) is seen in fig. 10, Plate 8, which was photographed from a 3 days-old vitally stained culture. The surrounding cells (M) are vitally stained polyblasts.

Usually with actively growing tumours, areas of liquefaction appear in the midst of the malignant cells, after 2 or 3 days. When the fibrin network breaks down, as has been described with other strains of tumours, the carcinoma cells are drawn aside by the contraction of the intact fibrin fibrils, leaving a clear area in which remain the vitally stained connective-tissue cells spread on the surface of the cover glass. Such a culture is shown in fig. 9, Plate 8, which depicts a 3 days-old culture treated with dye for 2 days; the remains of the unstained sheet of carcinoma cells (C) are seen partially surrounding the liquified area (L), with its vitally stained non-malignant cells (M), and the explant (X) at the bottom right-hand corner.

None of the serum cultures of this tumour has shown any out-growth of carcinoma cells. The explant itself becomes rounded off, and non-malignant connective-tissue cells migrate from it, along the under-surface of the cover-glass. Fig. 2, Plate 6, was drawn from a vitally stained 3 days-old serum culture of the same tumour as that shown growing in plasma in fig. 1 of the same plate. On the left side of fig. 2 is seen the rounded explant. The black dots scattered over it represent the dye droplets of vitally stained non-

malignant cells which are spread on the undersurface of the cover-glass ; the same type of cells have also wandered out into the medium. The centre of the explant frequently becomes necrotic, and stains diffusely blue, so also do clusters of dead cells around its periphery, while the cortical area is then comprised of living carcinoma cells, which contain no dye droplets.

The polyblasts and macrophages, which grow out from explants in serum, exhibit a wide variation in form as can be seen in figs. 6 and 10, Plate 8. All these cells were photographed in the living condition, and have been vitally stained by the trypan blue added to the cultures. Fig. 6 shows cells in the act of migrating from the explant, while in fig. 10 most cells (M) are spindle-shaped and superficially resemble fibroblasts.

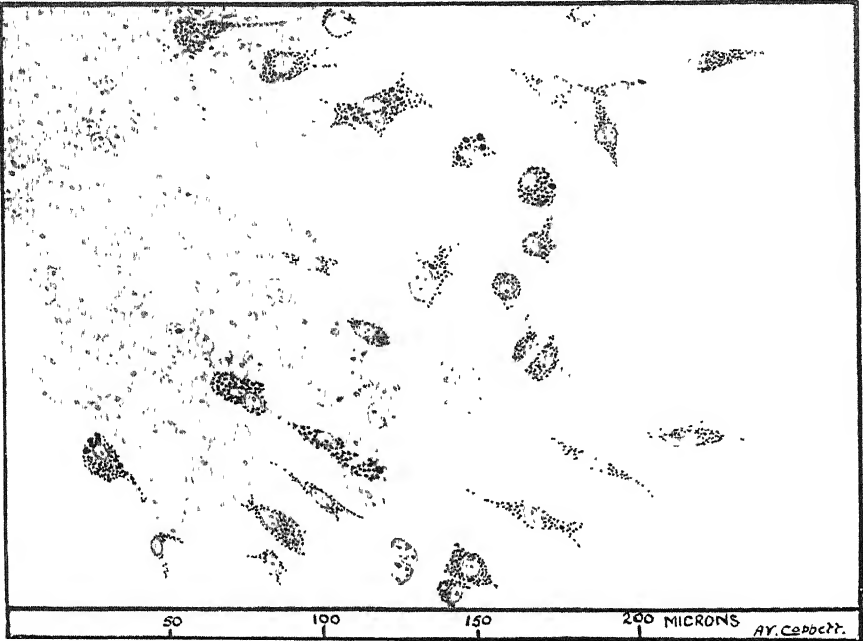
There are indications that the non-malignant cells when spread on the cover-glass in serum cultures are more sensitive to injury than the carcinoma cells, which, as I have pointed out, remain in the explant. In a few cultures to which rather more than the usual amount of trypan blue was added the non-malignant cells were killed, while the carcinoma cells within the explants remained uncoloured by the dye, thus indicating that they were still living. Also in some infected cultures the non-malignant cells on the side of the infection were killed, but the malignant cells in that neighbourhood were not.

Although we have never seen carcinoma cells grow out from the explants in serum cultures yet, by cutting serial sections, these cells can be seen actively dividing. Fig. 7, Plate 8, is a photomicrograph of a fixed and stained explant from a 2 days-old serum culture, to which trypan blue was added on the second day. Some vitally stained polyblasts were present in the section but, except for a few coloured phagocytosed particles, the carcinoma cells were free of trypan blue. The area represented in fig. 7 contains six carcinoma cells in mitosis. They are indicated by arrows.

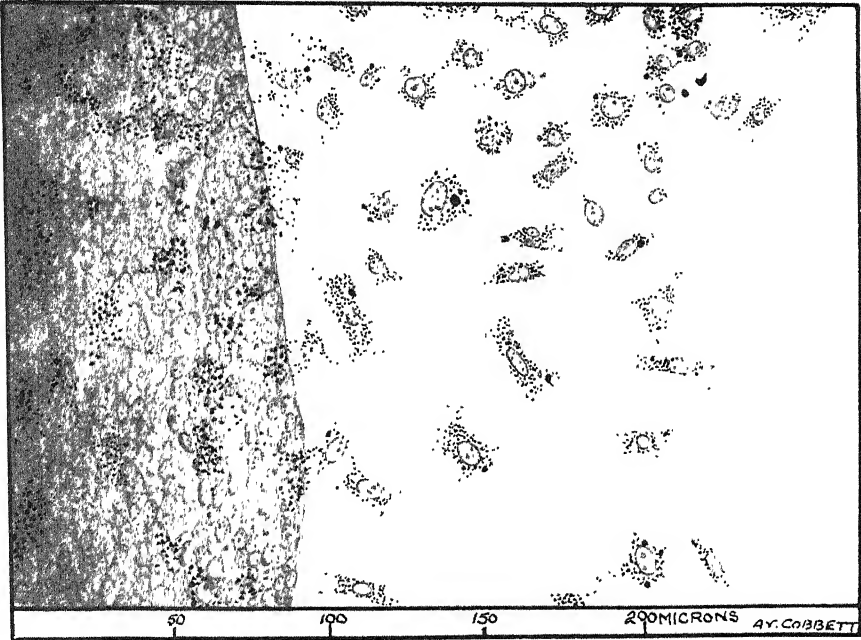
That the carcinoma cells of the rounded explants of serum cultures are capable of growing as sheets of cells, when the cells are provided with a suitable support has been shown in the following manner. The explants of 7 days-old serum cultures have been washed in Ringer's solution, then explanted in plasma. After 24 hours a drop of trypan blue has been added to the cultures. The carcinoma cells have grown as a sheet, and have not segregated the dye. Many of them contain phagocytosed dye particles varying in size and shape, easily distinguishable from the granulation in true vital staining (segregation).

5. Growth of the Mammary Carcinoma 206 in Plasma and in Serum.

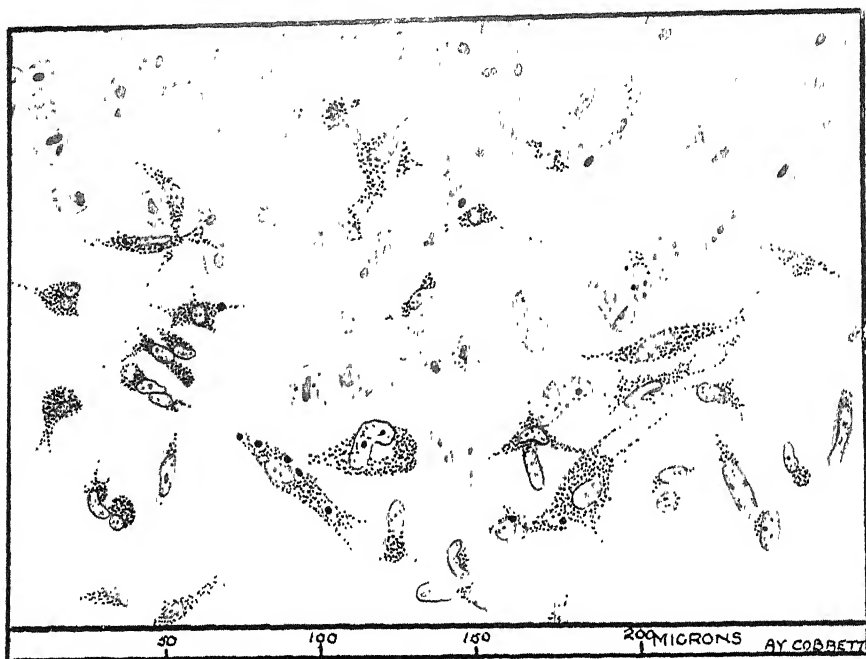
The carcinoma 206 grows at a similar rate to carcinoma 63 *in vivo*, but unlike it, commonly regresses. Both tumours have the same type of dedifferentiated



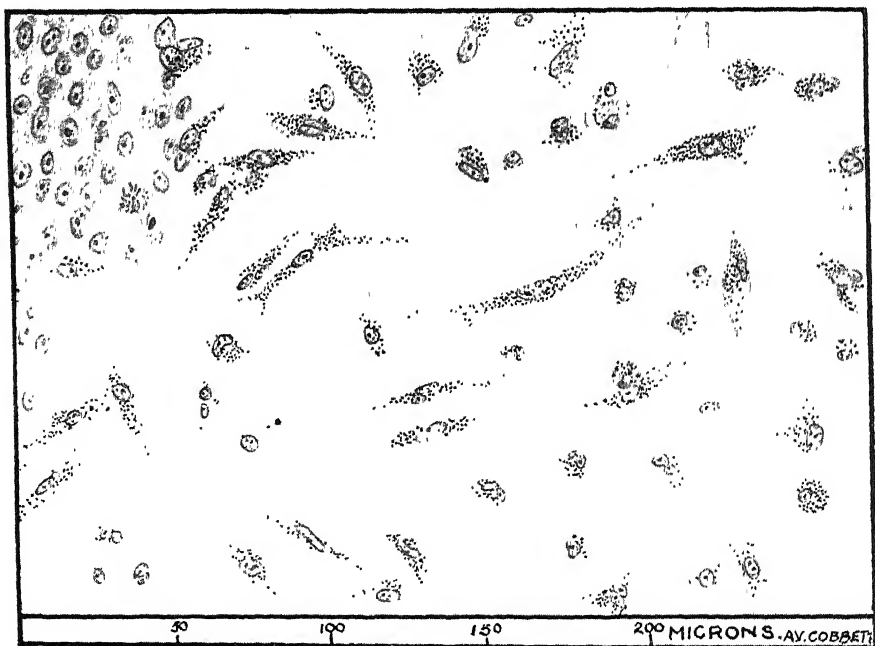
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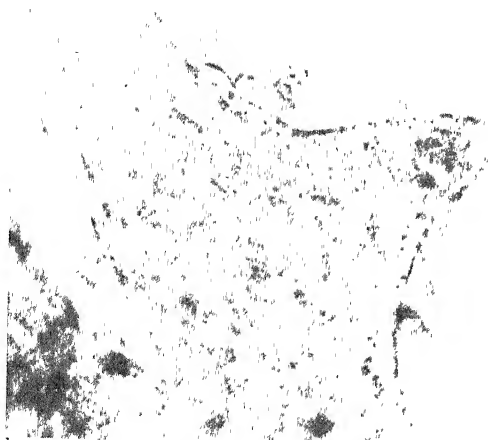


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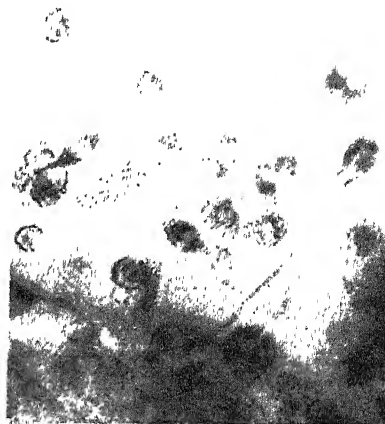


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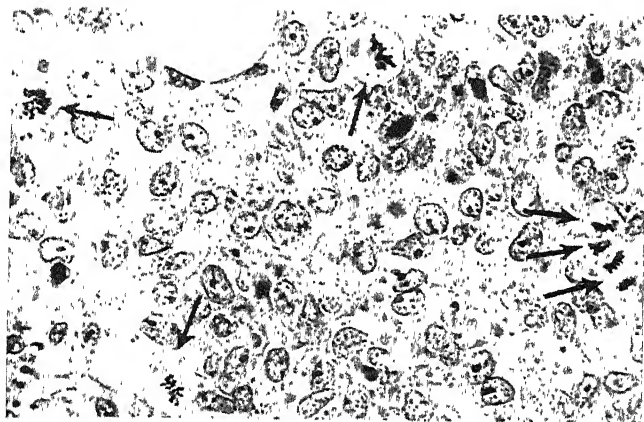




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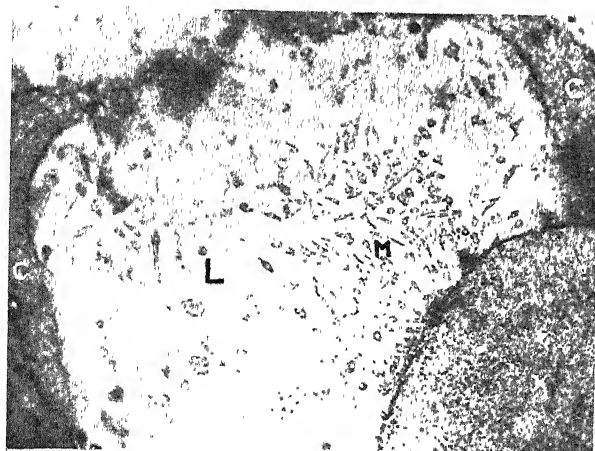
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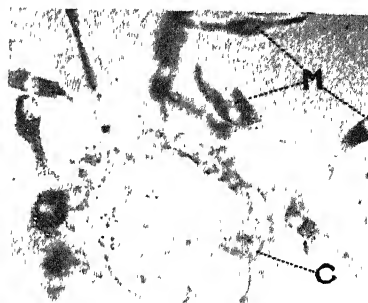
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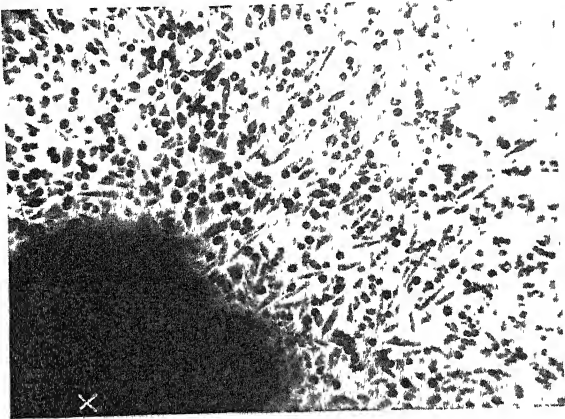
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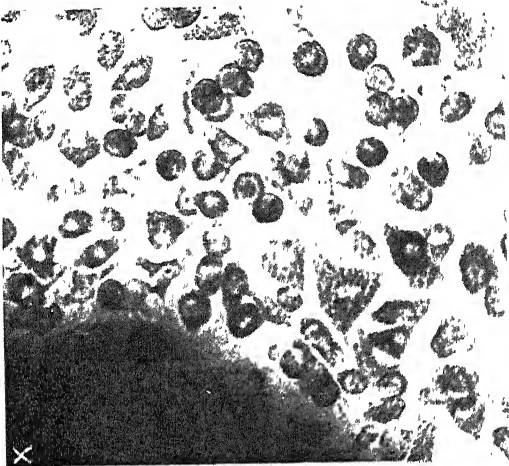
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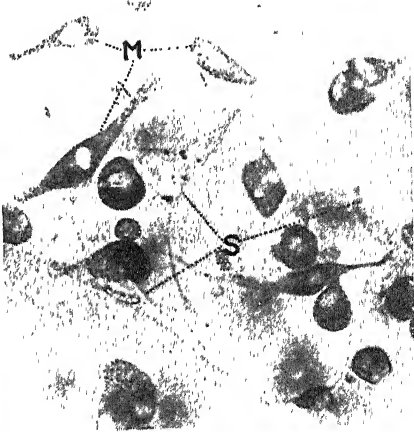
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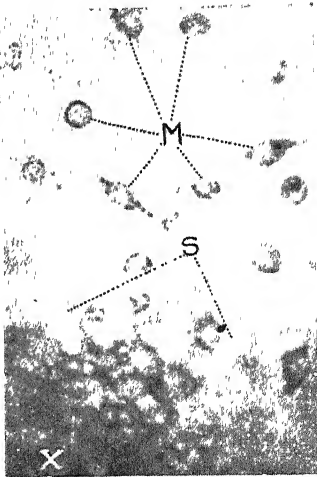
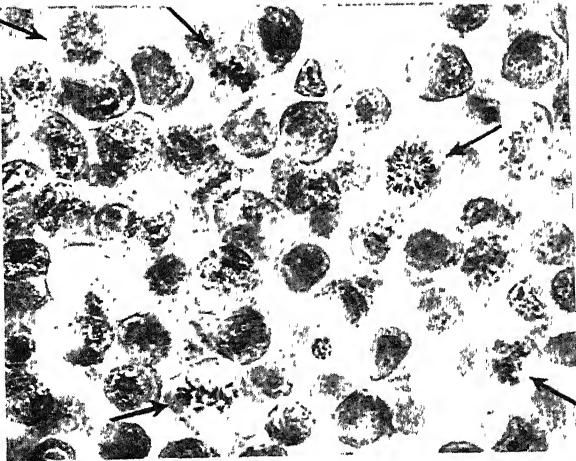
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cells. When grown in plasma the malignant cells of this tumour are less coherent than those of tumour 63, but form the same kind of sheet-like outgrowths. In liquefied cultures, after the sheet of carcinoma cells has collapsed, small rounded islands of malignant cells often remain in the fluid area amongst the numerous non-malignant cells, which also extend out into the intact plasma around the periphery of the culture.

A part of the sheet of carcinoma cells of a living 2 days-old plasma culture is seen more highly magnified in fig. 8, Plate 8. The carcinoma cells (C) are out of focus, since the sheet-like growth is at a lower level to the polyblasts and macrophages (M), which are shown wandering out into the medium.

The difference between the growth of this tumour in serum and in plasma is particularly striking. Serum cultures frequently show an extensive migration of non-malignant cells, which stain intensely with trypan blue. Fig. 11, Plate 9, depicts such a culture which was photographed in the living condition when 4 days old, and after it had been subjected to the action of trypan blue for 2 days. Not a single unstained carcinoma cell could be distinguished outside the explant. Living vitally stained non-malignant cells of another 3 days-old serum culture are shown more highly magnified in fig. 13, Plate 9.

In a very few serum cultures we have seen what looks like a small outgrowth of carcinoma cells, which remain uncoloured after adding trypan blue, but in none of these cultures have the carcinoma cells given rise to a sheet-like outgrowth, as occurs in plasma. It is possible that some substances may exude from the explant, and by coagulating in the serum provide a small support for the carcinoma cells, or a little fibrin may remain in the serum as Momigliano-Levi (1932) found.

Occasionally non-malignant cells in serum cultures of this tumour have been seen growing on two different planes, viz., on the undersurface of the cover glass in the usual manner, and again on some supporting structure lower in the drop. The latter may remain after the serum drop has been allowed to run to the edge of the cover glass, but on fixing such cultures these cells always become dislodged, so that we have not been able to retain them in permanent preparations.

6. Growth of the Crocker Sarcoma in Plasma and Serum.

The Crocker sarcoma grows rapidly both *in vivo* and *in vitro*. The behaviour of the normal and malignant cells of this tumour in plasma cultures has been described in previous papers (Ludford, 1932, *b*, 1932, *c*). Fig. 3, Plate 7, is

a drawing of a part of the margin of a 3 days-old culture to which trypan blue was added on the second day. The non-malignant connective-tissue cells are seen filled with dye droplets. These are absent from the sarcoma cells, which are also characterised by larger nuclei and relatively larger nucleoli. Fig. 14, Plate 9, is a photomicrograph of a living 7 days-old culture to which dye was added on the second day. It shows the same distinction between the dark vitally stained non-malignant cells (M) and the malignant ones (S).

Liquefaction of the plasma clot is followed, as in the carcinoma cultures, by collapse of the outgrowth of malignant cells, and survival of the vitally stained connective-tissue cells, which remain spread out on the undersurface of the cover glass.

Serum cultures of this tumour exhibit an entirely different type of growth. As with the carcinomata already described the only types of cells which wander out from the explants in any number are the non-malignant cells. The malignant cells within the explants tend to round off, and groups break away peripherally, and float out in the medium. Fig. 4, Plate 7, is a drawing of a serum culture of the same tumour, the growth of which in plasma is shown in fig. 3 above. Both figures were drawn from cultures of the same age, to which trypan blue was added at the same time. On the left of fig. 4 are seen the rounded sarcoma cells around the edge of the explant. One cell is dividing. The outgrowth consists entirely of vitally stained non-malignant cells adherent to the undersurface of the cover glass.

In a few instances we have had plasma cultures in which liquefaction has occurred very early, within the first 24 hours; under such conditions the growth is like that in serum. The only cells to wander out from the explant are the non-malignant ones, while the sarcoma cells around the edge of the explant become rounded off.

In less than half a dozen of 40 serum cultures small outgrowths from the explants of a few sarcoma cells have been seen. They are possibly the result of a little fibrin being present in that area. Isolated flattened sarcoma cells are also occasionally seen amongst the vitally stained connective-tissue cells. Rarely are they spread out to the same extent as in plasma, also they appear to be but loosely adherent. Usually the act of mounting the culture in Ringer solution for cytological examination is sufficient to cause them to become rounded, and even to float off the cover glass. It is believed that these cells have floated away from the explant and become secondarily attached.

7. *Growth of the Sarcoma 37 in Plasma and in Serum.*

The sarcoma 37 is very similar to the Crocker tumour, and exhibits the same type of growth in plasma cultures. Serum cultures exhibit a more extensive disintegration of the explants than occurs in those of the Crocker sarcoma. There is the possibility that this is the result of the cells of the sarcoma 37 being in a more active state during the period over which the experiments were performed. Fig. 12, Plate 9, shows the explant of a living 2 days-old culture to which trypan blue was added on the second day. The sarcoma cells have become rounded. Many of them appear black in the photomicrograph; in the culture they were stained diffusely blue, thus indicating that they were necrotic. This figure illustrates the way in which the explant has disintegrated. Groups of cells become completely detached and float away from the explant, though some may again become lightly attached to the cover glass. In a few cultures short protoplasmic processes have been seen extending from rounded cells to the surface of the cover glass. Most of the groups of sarcoma cells float freely in the serum, and the remains of the explant are but lightly attached, so that unless special precautions are taken in the process of fixation it becomes detached.

Mitosis is of common occurrence in the rounded sarcoma cells before they float free, especially during the first 24 hours after explantation. Fig. 15, Plate 9, is a photomicrograph of a 20 hours-old culture, which was fixed in "Susa," and stained with Rawitz carmine; there are five mitotic figures indicated by arrows. Serum cultures of this type would afford an excellent means for the study of chromosomes in sarcoma cells.

Amongst the disintegrating explants of serum cultures are to be seen the non-malignant cells adherent to the undersurface of the cover glass. Fig. 16, Plate 9, shows some of these cells (M) vitally stained with trypan blue in a 2 days-old culture. Rounded sarcoma cells (S) of the explant are seen out of focus, since they are not flattened on the glass in the same manner as the non-malignant cells.

8. *Discussion.*

(i) *The Bearing of these Experiments on the Nature of the Plasma Membrane of the Cancer Cell.*—The experiments described above have revealed a marked difference in the mode of growth of a tumour when explanted in plasma and in serum. While both malignant and non-malignant cells migrate from the explant in plasma, only the latter consistently wander out in serum. Malignant cells thus need a fibrin network for their support, while non-malignant cells

can glide along the undersurface of the cover glass. This fact, together with the observations described more fully elsewhere (Ludford, 1932, *c*) that when a plasma culture of a tumour undergoes liquefaction the outgrowth of malignant cells collapses, confirms the suggestion previously advanced that the plasma membrane of malignant cells is less adhesive than that of non-malignant cells.

It is conceivable that a difference in the chemical constitution of the plasma membrane of the malignant cell may be responsible both for the alteration in its adhesiveness, and also its failure to segregate acid dyes as the result of an altered permeability. A greater amount of fats or lipins in the plasma membrane might prevent cells from adhering to glass, and at the same time stop the penetration of the water soluble acid dyes, such as trypan blue and vital new red.

(ii) *The Non-malignant Cells of Tissue Cultures of Tumours, their Origin and Fluctuations in Number.*—It has been shown previously (Ludford, 1932, *a*) that the tumour 206 exhibits variations in its growth in tissue cultures according to its state of growth *in vivo*. Thus rapidly growing tumours in young mice when explanted grow as sheets of carcinoma cells: tumours growing slowly *in vivo*, on explantation, exhibit considerable outgrowths of non-malignant cells—monocytes, polyblasts, macrophages and fibroblasts, while cultures of regressing tumours usually consist entirely of non-malignant cells. Since only these cells wander out freely in serum cultures it is not surprising to find that the best outgrowths of cells occur in this medium when tumours are explanted at a time when they are not growing at their best *in vivo*. It has not been uncommon to find that tumours which on being explanted in serum have given rise to most extensive outgrowths have yielded in plasma cultures but a poor growth of malignant cells.

Before investigating the state of affairs within the explants, it was difficult to account for all the non-malignant cells seen in some cultures. It was found, however, that the non-malignant cells multiply by mitosis within the explants. In cultures of the carcinoma 27 many more mitoses were observed in these cells than in the malignant ones (Ludford, 1932, *c*).

Russell (1908) and Woglom (1912) showed that on transplanting a tumour the stroma cells of the graft die, and whether growth will proceed, or not, depends upon whether the tissues of the host supply a new stroma. This discovery, together with the fact that it has been demonstrated by da Fano (1912) and others, that infiltration by mononuclear cells is characteristic of regressing tumours, points to the conclusion that the non-malignant cells,

which wander out from explants of transplantable tumours *in vitro*, are derived from the animal in which the graft has been grown. No transformation of malignant cells into cells such as those which segregate trypan blue has been observed *in vitro*.

(iii) *The Serum Culture Technique, and the Search for Substances capable of exerting a specific Destructive Action upon Cancer Cells.*—Although earlier workers had expressed the view that serum inhibited growth *in vitro*, Lumsden (1924) was able to show that it is possible to obtain vigorous growth in this medium by paying due regard to its p_H which must not exceed 7·7. He showed that heterologous serum was as effective as homologous, since he obtained good cultures of young rat kidney in rabbit serum. Later des Ligneris (1928) made an extensive study of the growth of different tissues in serum, and came to the conclusion that “mammalian cells other than trephocytes do not grow in serum cultures.” He includes as trephocytes the cells of the reticulo-endothelial system, including endothelial cells of the blood and lymph vessels, and fibroblasts.

Lumsden (1931) has employed his serum method for the study of tumour immunity *in vitro*. Without any methods for distinguishing normal from malignant cells, such as those we have used, he failed to discover that the cells which migrate from tumour explants in serum are non-malignant cells. Consequently when he added anti-sera to his cultures, and killed off these non-malignant cells, he erroneously concluded that he had demonstrated a specific destructive action upon cancer cells. None of the photomicrographs of his experiments shows other than the destruction of non-malignant cells. This can readily be appreciated by comparing figs. 9, 11, 18, 24 and 25 of tumour 63 in the recent review of his work, in the ‘American Journal of Cancer’ (1931), with figs. 6 and 10 of Plate 8, of the present paper where are represented vitally stained cells of the polyblast and macrophage types of the same tumour. A similar error in identifying the cells which grow out from explants in saline media was made by Drew, although he realised that “growth in serum is specially unsuitable for tumour study.”

On the basis of our recent investigations it becomes possible to establish certain tests for any substances, for which a specific destructive action on cancer cells *in vitro* is claimed :—

- (1) When added to a young actively growing plasma culture such a substance should be able to kill off the malignant cells and leave the non-malignant cells unaffected. The latter can be distinguished by their

general cytological characters, as well as by their reactions to acid and basic dyes.

- (2) When added to a young healthy serum culture such a substance should be capable of destroying the malignant cells within the explant, while leaving intact the cells which have wandered out from it, since these are non-malignant cells. The cells of the explant can be examined cytologically by cutting it in serial sections, by partially crushing it, or by teasing it apart.

For such tests old cultures should not be used, since as has been previously mentioned, if plasma cultures are left without sub-culturing the malignant cells frequently die, leaving only non-malignant cells of the macrophage type.

In seeking for differences between malignant and non-malignant cells it is obvious that malignant cells should be compared with their non-malignant prototypes: sarcoma cells with fibroblasts, and mammary-carcinoma cells with mammary-gland cells. Further, should a substance be found which had a specific destructive action upon malignant cells *in vitro*, it does not necessarily follow that it would have the same action *in vivo*, or *vice versa*.

9. Summary.

- (1) In tissue cultures of tumours there are present—

- (a) the malignant cells ;
- (b) non-malignant cells, consisting of cells of the stroma, and cells of the monocyte—macrophage series, which vary in number according to the extent of the resistance, which the animal from which the tumour was removed, had opposed to the malignant growth.

- (2) The malignant cells can be distinguished from the non-malignant cells by the addition of trypan blue to the cultures, since the former do not segregate the dye like the latter. The two types of cells are further distinguishable by vital staining with a basic dye, such as neutral red, and by their general cytological characters, such as size of nuclei and nucleoli, and cytoplasmic vacuolation.

- (3) In plasma cultures both malignant and non-malignant cells migrate from the explants (carcinoma, fig. 1, Plate 6 ; sarcoma, fig. 3, Plate 7).

- (4) In serum cultures only non-malignant cells wander out from the explants (carcinoma, fig. 2, Plate 6 ; sarcoma, fig. 4, Plate 7).

(5) In serum cultures the explants of carcinomata soon become rounded, fig. 2, Plate 6. The carcinoma cells inside the explants divide mitotically, fig. 7, Plate 8.

(6) In serum cultures the explants of sarcomata tend quickly to disintegrate—the sarcoma cells rounding off—and later float freely in the medium, fig. 12, Plate 9. Mitosis occurs in rounded sarcoma cells, fig. 15, Plate 9.

(7) The non-malignant cells which wander out in serum cultures exhibit a wide variety of forms, but they all stain vitally with trypan blue, figs. 6 and 10, Plate 8, and figs. 11 and 13, Plate 9.

(8) The outgrowth of non-malignant cells in serum cultures is usually greatest when tumours are explanted at a time when they are not growing at their best *in vivo*. Plasma cultures made at the same time do not exhibit good growths of malignant cells.

(9) It is suggested that the different behaviour of malignant cells in plasma, and in serum, is the result of an alteration in their plasma membrane such that they are unable to adhere to glass, though able to use the fibrin network of a plasma clot as a support for their movement. This may be brought about by a greater amount of fats or lipins in the plasma membrane. To such an alteration may be attributed also the failure of malignant cells to segregate the water soluble acid dyes, since these may be unable to penetrate such a membrane.

(10) The results of this research render invalid conclusions drawn concerning the specific destructive action of immune sera on cancer cells in serum cultures (p. 259). Tests for any further claims of this kind are formulated (pp. 259 and 260).

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DESCRIPTION OF PLATES.

All the cultures shown in the figures were grown either in rat plasma, or rat serum. The age of each culture, and the time it had been exposed to the action of the dye are indicated in an abbreviated form at the end of each legend. The figure before the letter "B" denotes the age in days of the culture at the time trypan blue was added to it; the figure after the letter "B" denotes the number of days the dye remained on the culture. Thus, 2B1 implies that the culture was 2 days old when trypan blue was added, and the dye had been present for 1 day.

Explanation of Lettering.

C., carcinoma cells. L., liquefied area in plasma culture. M. non-malignant connective-tissue cells—monocytes, polyblasts and macrophages. S., sarcoma cells. X. explant.

PLATE 6. CARCINOMA 63.

Camera lucida drawings of "Susa"-fixed preparations, post-vitally stained with Rawitz carmine. The blue droplets of the vitally stained non-malignant cells are represented by black dots.

FIG. 1.—Plasma culture showing a narrow sheet-like growth of carcinoma cells containing no dye droplets, with scattered vitally stained non-malignant cells. The cell with the fine dye droplets near the centre of the figure is a fibroblast. 1B2.

FIG. 2.—Serum culture of the same tumour showing part of the rounded explant on the left, and the outgrowth of non-malignant vitally stained cells. 1B2.

PLATE 7. CROCKER SARCOMA.

Camera lucida drawings of "Susa"-fixed vitally stained cultures, post-vitally stained with Rawitz carmine. The blue droplets in the non-malignant cells are represented by black dots.

FIG. 3.—Sarcoma cells and vitally stained non-malignant cells on the margin of the growth in a plasma culture. The explant lies above the top of the figure. 1B2.

FIG. 4.—Vitally stained non-malignant cells, which have wandered out from an explant in serum of the same tumour as fig. 3. Rounded sarcoma cells on the margin of the explant, one of which is in mitosis, are seen on the left of the figure. 1B2.

PLATE 8.

FIG. 5.—Living plasma culture of carcinoma 63 showing sheet growth of unstained malignant cells. 1B2. $\times 280$.

FIG. 6.—Living vitally stained non-malignant cells in process of migrating from the explant (X) of a serum culture of carcinoma 63. 1B1. $\times 280$.

FIG. 7.—Section of the fixed and stained explant of a 2 days-old serum culture with carcinoma cells in division indicated by arrows. Carcinoma 63. 1B1. $\times 520$.

FIG. 8.—Part of the margin of the sheet of carcinoma cells (C) in a living 2 days-old plasma culture, showing also the polymorphous non-malignant cells (M). Carcinoma 206. $\times 280$.

FIG. 9.—Living liquefied plasma culture of carcinoma 63. In the liquid area (L) are numerous vitally stained non-malignant cells (M). On either side are the remains of the sheet of unstained carcinoma cells (C) (explant—X). 1B2. $\times 70$.

FIG. 10.—A small plaque of living carcinoma cells (C) surrounded by vitally stained non-malignant cells (M) in a plasma culture of carcinoma 63. 1B2. $\times 280$.

PLATE 9.

- FIG. 11.—Outgrowth of vitally stained non-malignant cells in a living serum culture of carcinoma 206. (X—explant.) 2B2. $\times 70$.
FIG. 12.—Disintegration of the explant in a serum culture of sarcoma 37, showing rounded living sarcoma cells, and dead cells stained diffusely appearing black in the photomicrograph. 1B1. $\times 70$.
FIG. 13.—Living vitally stained non-malignant cells in a serum culture of carcinoma 206, more highly magnified than fig. 11. 2B1. $\times 280$.
FIG. 14.—Unstained sarcoma cells (S) and vitally stained non-malignant cells (M) in a living plasma culture of the Crocker sarcoma. 1B6. $\times 280$.
FIG. 15.—Rounded sarcoma cells in a fixed and stained preparation of the disintegrated explant of a serum culture. Sarcoma cells in mitosis are indicated by arrows. 20 hours-old culture. Sarcoma 37. $\times 560$.
FIG. 16.—Vitally stained non-malignant cells (M) in a living serum culture of sarcoma 37. Rounded sarcoma cells (S) are floating freely in the serum, below the non-malignant cells (explant—X). 1B1. $\times 280$.
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572 . 7 : 569—8 *Sinanthropus*

*On the Endocranial Cast of the Adolescent Sinanthropus Skull.**

By DAVIDSON BLACK, F.R.S.

(Received September 9, 1932).

[PLATES 10-15].

The cast to be described in this paper is that of the adolescent skull specimen from Locus E of the Choukoutien deposit, which has already been the subject of detailed report (Black, 1931). Earlier it was anticipated that the endocranial cast of both this specimen and that of the adult skull from Locus D would be described together, but as circumstances have retarded the work of restoration on the latter specimen, the present report will no longer be delayed.

Cranial Capacity.

Under this heading in the description of the skull (*loc. cit.*, p. 45) it was estimated from the crude preliminary trial measurements that the endocranial volume of the restored specimen would be in excess of 1000 c.c. This prediction has, however, not been confirmed and the volume of the restored endocranial

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cast has now been determined with accuracy to be 964.4 ± 0.27 c.c. Since the accuracy of any determination of skull volume is largely dependent upon the method employed it may be of advantage to describe the technique by which the above result has been obtained.

The first crude estimates of volume were made by measuring the amount of water required to fill the unrestored negative cast made of the endocranial cavity. The results of these repeated measurements ranged between 980 c.c. and 990 c.c. as already recorded. A number of trial restorations of the base of the endocranial cast were then made and cast in plaster before the one which probably approximates most nearly to the correct form was selected. These various plaster casts were dried and waterproofed by impregnation with shellac under negative pressure. Their respective volumes were then determined in water by the displacement method in a cylindrical vessel just accommodating the specimens. As a result of repeated trials by two independent and qualified observers the volume in each approximated 900 c.c., it being impossible to obtain a more accurate reading of volume displacement in a vessel of the size necessary to accommodate one cast.

To overcome this difficulty a Negocoll cast was made of the restored endocranial cast which had been finally selected for description. This Negocoll was then melted and poured in cylindrical moulds which when set were of a size readily to be accommodated within graduated measuring cylinders. Twenty-five consecutive determinations of the volume of these Negocoll cylinders were then made in order to permit the calculation of the probable error.

It may be noted that when it is possible to make a Negocoll endocranial cast of a skull, the method outlined above will probably yield the most accurate volumetric results and in the present instance it was desirable to obtain a reliable figure. It is questionable, however, if aside from such special cases the information so obtained justifies the expenditure of time, for an accuracy to within ± 50 c.c. would seem to be sufficient for all ordinary purposes.

Though of a volume of but 964 c.c. the cranial capacity of the adolescent *Sinanthropus* skull falls well within the lower range of normal variability of this character in modern man. I have before me, for example, a copy made some years ago of the endocranial cast of the "Small New Britain Skull" from the series in the Museum of the Royal College of Surgeons. In this cast the volume approximates 900 c.c. and there is no evidence of any cranial abnormality. Professor Wood Jones also has recently drawn attention to the fact that among the Australian aborigines similarly small endocranial volumes

are by no means rare. It may be considered probable that endocranial volumes of 1000 ± 50 c.c. occur in mentally normal individuals among many other modern racial groups.

In view of his known usage of fire and of his ability in the manufacture of crude stone artifacts it was to be expected that the cranial capacity in *Sinanthropus* would not be found to differ markedly from that of some of his modern successors. On the other hand, the very crudity of his lithic culture (Teilhard de Chardin and Pei, 1932, p. 354) serves to imply a possible difference in the relative representations within his brain of those cerebral regions whose more advanced development makes possible to the modern savage his superior technique.

General Description of Cast.

It will be recalled that the whole of the vault of the adolescent *Sinanthropus* skull was preserved, but of the base there was missing much of the foramen magnum area, the whole basi-occipital and the ethmoid regions, together with most of the sphenoid element. Fortunately a part of the left ex-occipital, marked on its endocranial surface by the occipital sinus groove, was preserved and from the latter landmark and the anterior walls of the two jugular fossæ it has been possible to locate with some degree of probability the approximate position of the foramen magnum. The margin of the inferior petrosal sinus groove was preserved on the right side to some extent at its anterior end so the general trend of this channel backwards to the jugular fossa could be determined. That part of the cast representing the sella turcica is wholly conjectural as also are the medial limits of the middle cerebral fossæ.

The endocranial surface of the lateral part of the great wing of the left sphenoid together with much of that of the lesser sphenoidal wing is preserved so that a perfect cast of most of the region occupied by the left temporal cerebral lobe remains, from which the right side could be modelled. The greater part of the left frontal orbital endocranial surface was also preserved, making possible the approximate restoration of the corresponding area on the right. In the endocranial cast illustrated in natural size in the accompanying Plates 10 to 15, the restored portions are indicated in dark tone, having been painted an ochre colour in the cast photographed.

The relations of the various component bones of the skull vault to the underlying areas of the brain are especially clear on the cast since none of the definitive cranial sutures of this skull had begun to close. These suture lines and other landmarks of importance are indicated here diagrammatically in

figs. 1 to 6, the outlines of which have been drawn from the plate photographs and are reproduced at a reduced scale.

On the base may be distinguished the prominences and depressions caused by the chief bony endocranial irregularities of each temporal bone, viz., the internal auditory meatus, the eminentia arcuata, the fossa subarcuata, the endolymphatic aperture and the prominence caused on the exterior by the glenoid fossa.

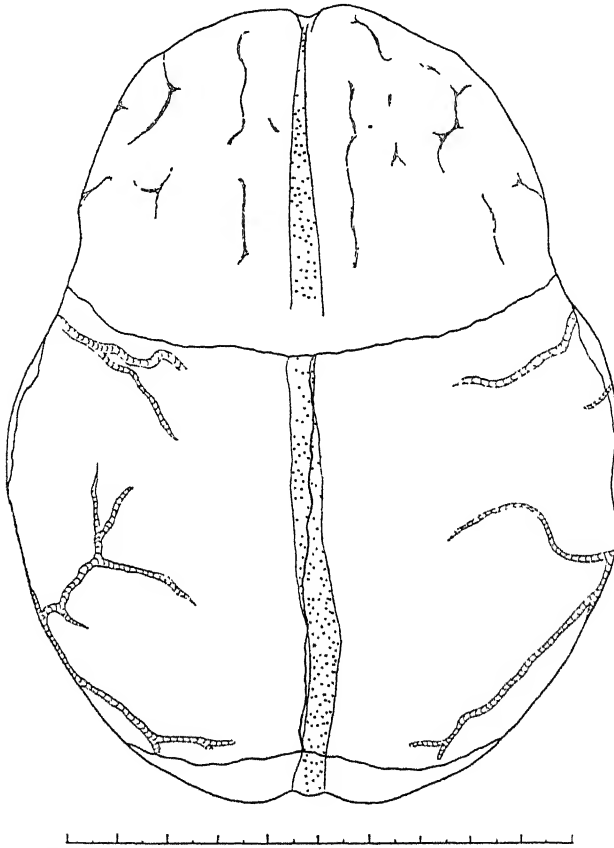


FIG. 1.—Diagrammatic drawing of norma verticalis view of cast. The details illustrated are indicated as follows: vascular sinus markings, stippled; vascular markings of middle meningeal vessels, curved line shading; sutures, fine continuous lines; probable course of sulci, heavy continuous lines. $\times 2/3$.

Vascular Markings.

Sinuses.—The site of lodgment of the superior longitudinal sinus begins as a narrow but clearly marked groove between the rostral cerebral poles. It

gradually increases in breadth, becomes shallower as the region of the bregma is approached and for a short distance rostral of the latter point it is scarcely to be distinguished on the cast. At the bregma its course deviates somewhat to the left and for a short distance backward its course lies wholly on the left parietal. Regaining the mid-line, this well-marked structure preserves its

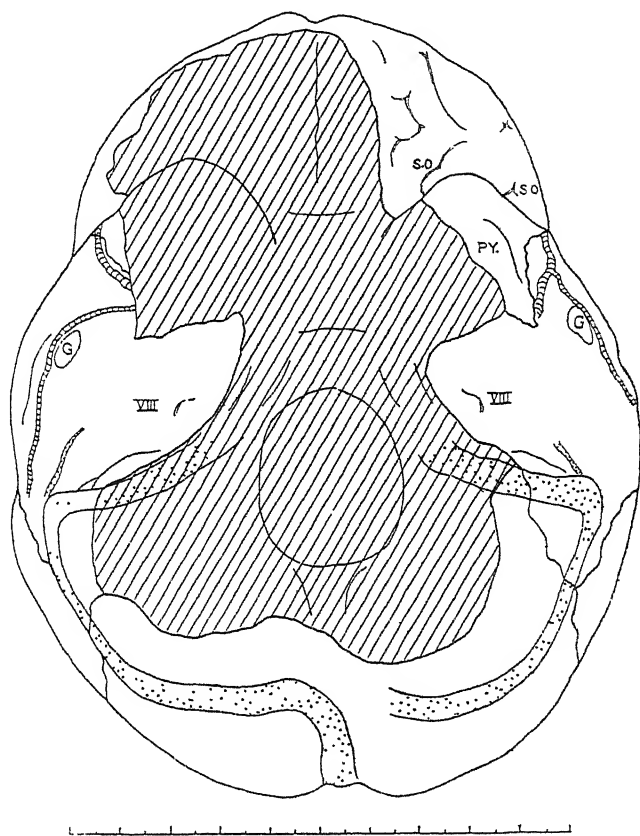


FIG. 2.—Diagrammatic drawing of norma basalis view of cast. The details illustrated are indicated as follows: restored portion of cast, oblique line shading; other conventions as in fig. 1. Abbreviations: G, depression caused by external form of glenoid fossa; PY, area pyriformis; SO, lateral and medial extremities of groove probably representing the sulcus orbitalis; VIII, site of internal auditory meatus. $\times 2/3$.

mid-sagittal course till the obeliatric region is reached. About 25 mm. rostral of the lambda it deviates to the right and in the last part of its parietal course lies wholly upon the right side.

At the confluence the superior longitudinal sinus groove becomes continuous in a graceful curve with the right occipital sinus as usually occurs in

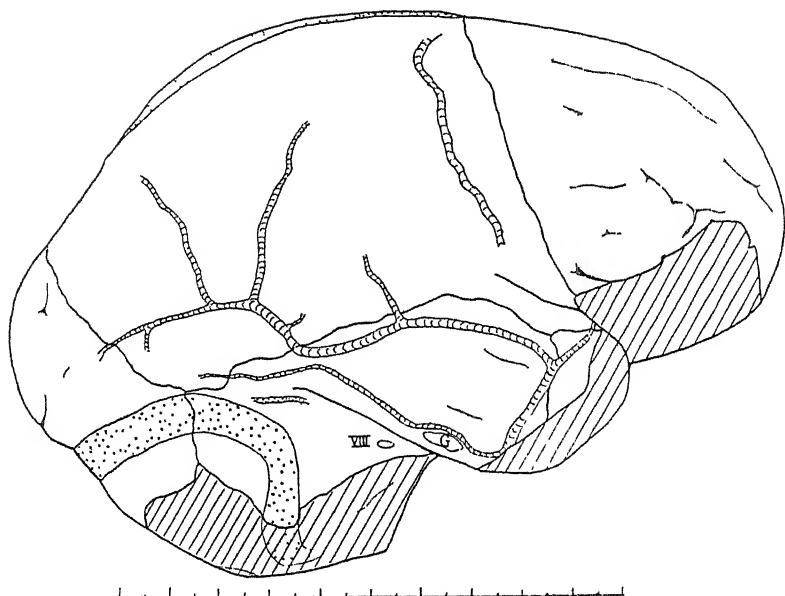


FIG. 3.—Diagrammatic drawing of right norma lateralis view of cast. Conventions and abbreviations as before. $\times 2/3$.

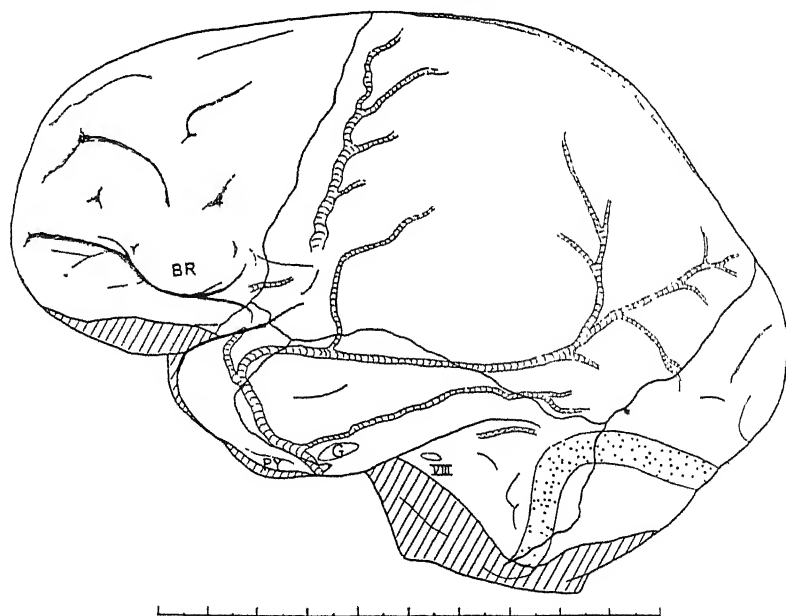


FIG. 4.—Diagrammatic drawing of left norma lateralis view of cast. Abbreviations: BR, inferior frontal prominence representing Broca's convolution. Other abbreviations and conventions as before. $\times 2/3$.

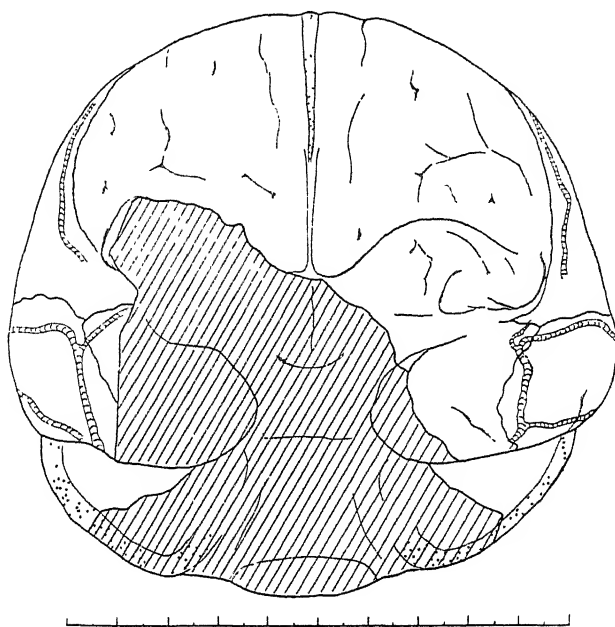


FIG. 5.—Diagrammatic drawing of norma frontalis view of cast. Conventions as before.
 $\times 2/3$.

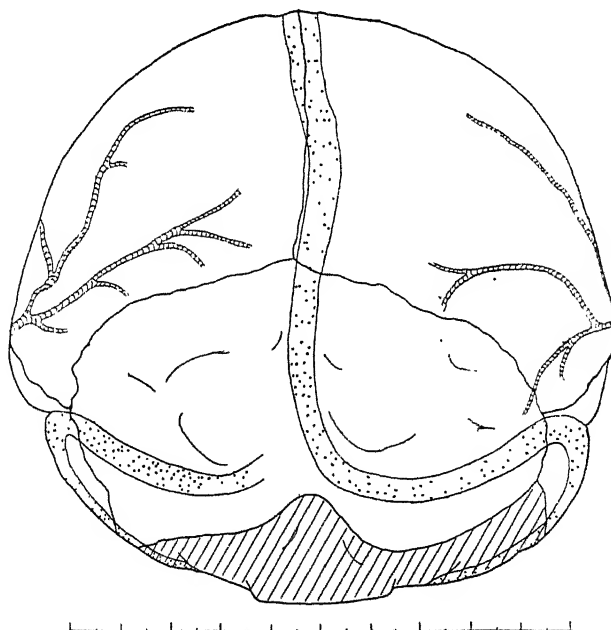


FIG. 6.—Diagrammatic drawing of norma occipitalis view of cast. Conventions as before.
 $\times 2/3$.

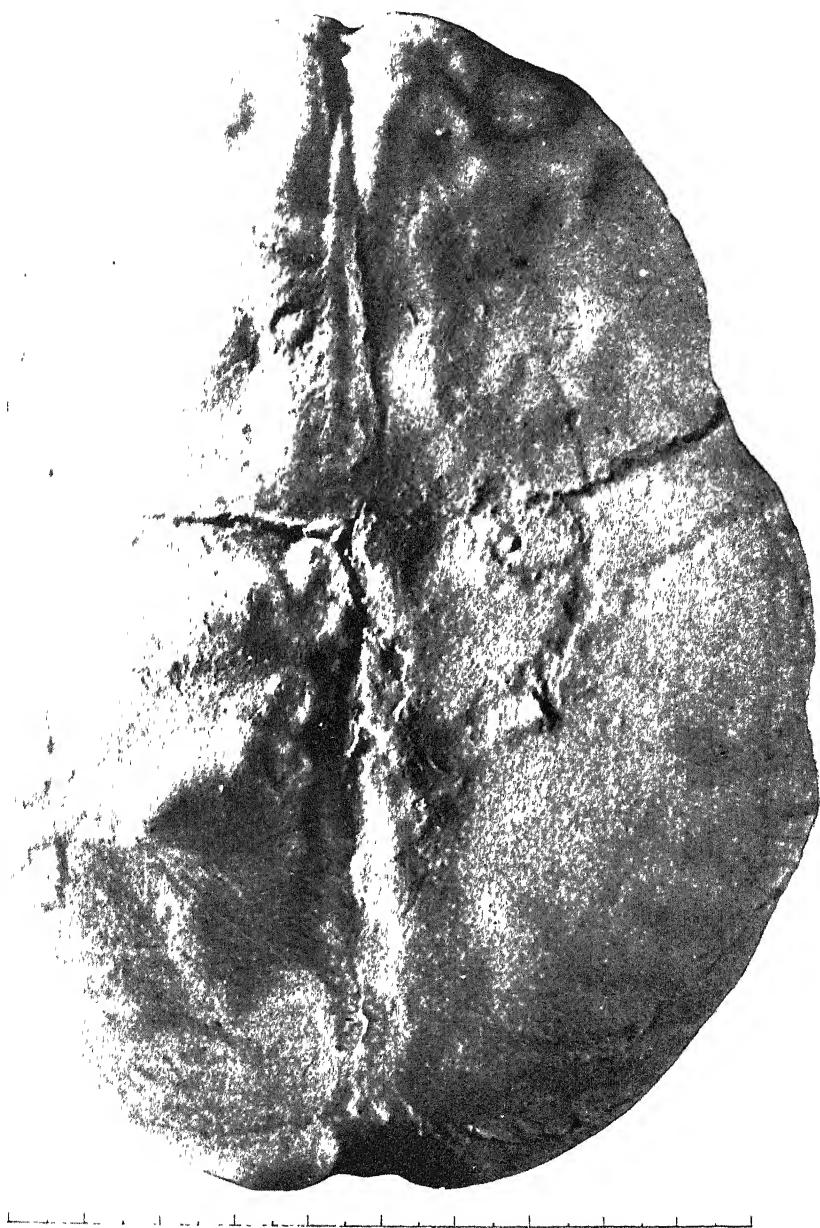
modern man. The right occipital sinus then sweeps forward and downward in its characteristic sigmoid course to the jugular region. On the left side the occipital sinus groove at its origin is separated from the confluence by a shallow ill-defined groove. The limits of the sinus groove rapidly become sharply defined and it courses laterally at a somewhat higher level than its fellow of the opposite side.

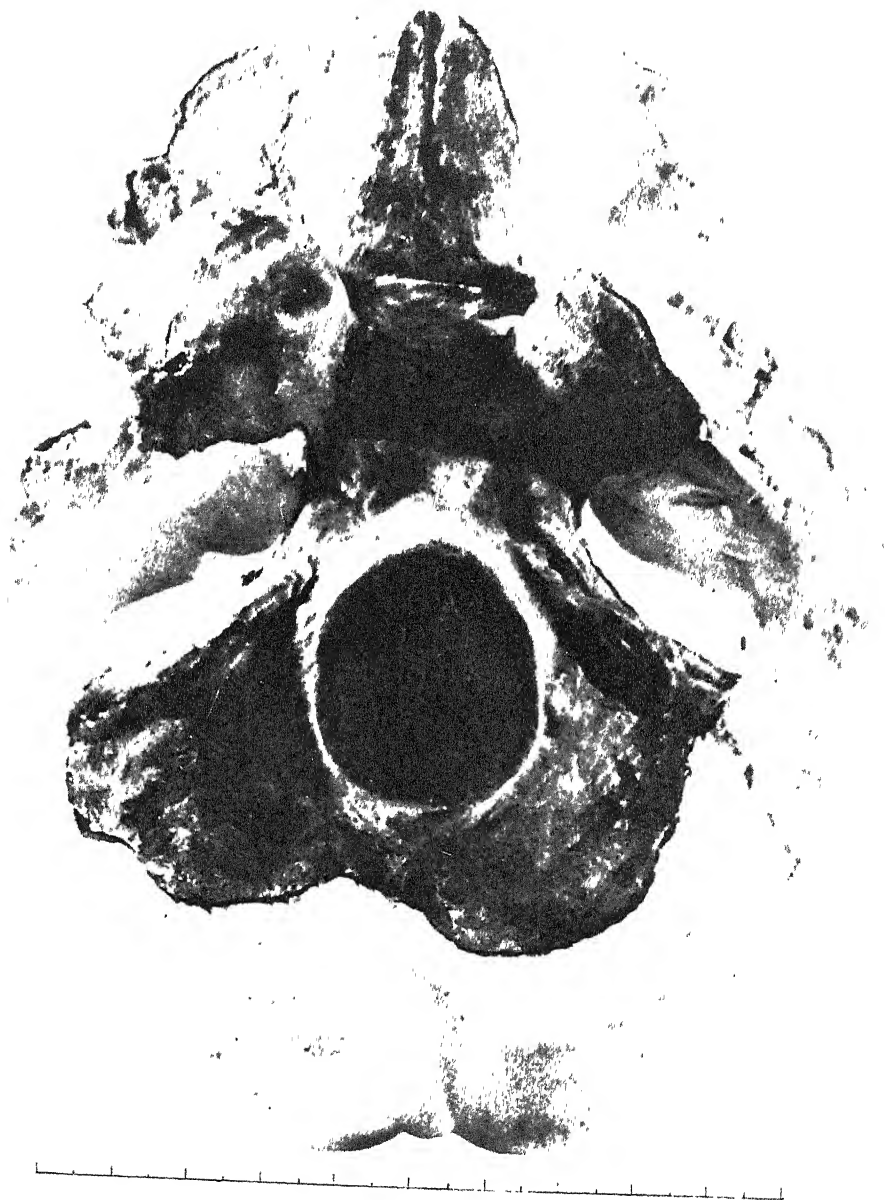
The superior petrosal sinus grooves on both sides are readily to be distinguished, each joining its corresponding occipital sinus groove at the sigmoid curve. Three quite large but somewhat shallow and ill-defined fossæ apparently for the lodgment of Pacchionian bodies are evident about the left pars bregmaticus region and two, one on either side, occur near the pars verticis region of the sagittal suture.

Meningeal Vascular Grooves.—The distribution pattern of the chief branches of the middle meningeal vessels is very clearly marked upon the cast and has been indicated diagrammatically in the text figures. On both sides the vascular pattern conforms essentially to Giuffrida-Ruggeri's *Type IIa* (1912) in which the ramus bregmaticus is a well-marked unit which with a main ramus lambdicus arises from the common vertical branch, practically on the sphenotemporal border. Unlike the *Type IIa* pattern the ramus præobelicus is derived on both sides in *Sinanthropus* from the ramus lambdicus. The ramus obelicus arises from the main ramus lambdicus on both sides. On both sides also there is to be distinguished a small but well-marked meningeal vascular groove arising from the parent stem shortly after the latter has gained the inferior temporal surface and coursing backwards across the inferior temporal region to the region of the asterion.

Cerebellar Region of Cast.

Though only the upper part of the endocranial surface of the cerebellar fossa has been preserved, some information concerning the development of this region of the brain can be deduced from its study. The endocranial contact surface of the right cerebellar hemisphere is preserved in this specimen to a lesser extent than on the left, but it would seem that the right hemisphere fossa had been definitely the larger and more prominent of the two. On both sides the beginning of the faint groove corresponding to the sulcus horizontalis magnus cerebelli may clearly be seen and felt on the cast. Since the cerebellum is an organ concerned chiefly in the elaboration of homolateral tonic, sthenic and static impulses of motor control, its apparent asymmetry in favour of the

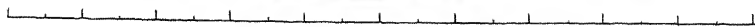
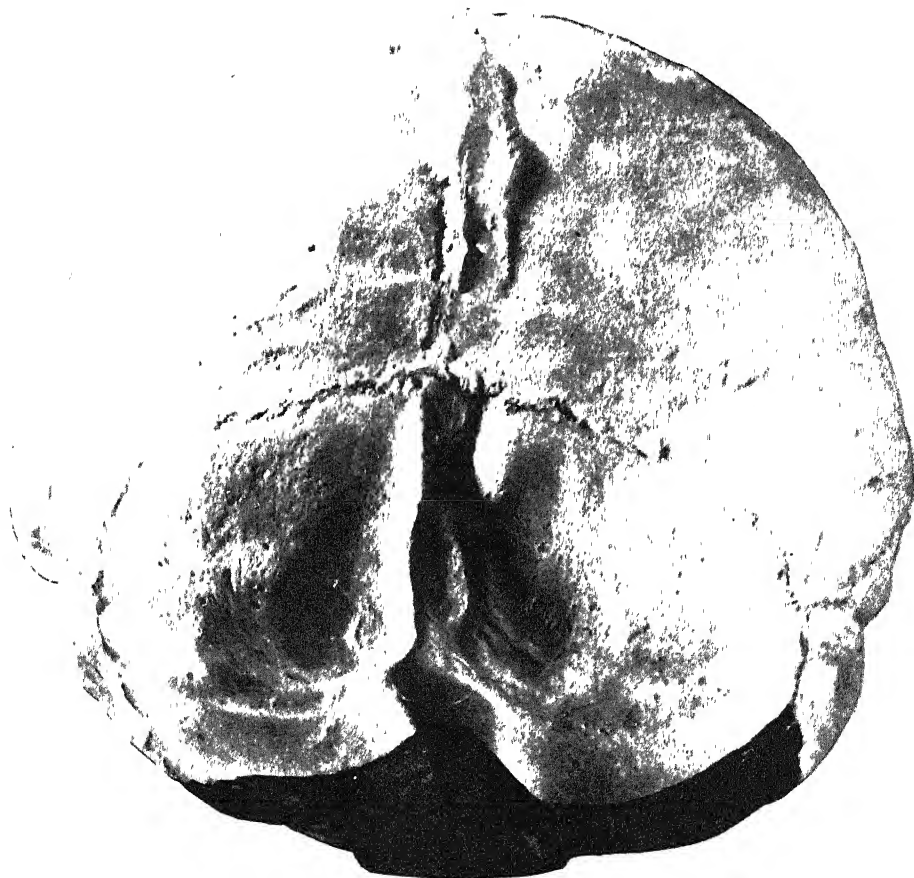












right side may not be without significance, but a consideration of this evidence will be deferred till after the proportions and morphology of the cerebral regions of the cast have been described.

Cerebral Region of Cast.

The general contours of the cast on the whole exhibit a remarkable degree of bilateral cerebral symmetry, but a closer examination of its proportions reveals the fact that the left cerebral hemisphere in its occipital and parietal regions is the larger, fuller and more rounded of the two. This fact may be gleaned by an examination of the photographs reproduced in the plates, but it becomes much more evident from an inspection of the cast itself when the latter is placed in positions differing from the standard ones in which the cast was photographed. In contrast to the occipital and parietal areas, the mid-frontal region on the left is slightly flatter and less prominent than that on the right, although the left inferior frontal area is more prominently developed than its counterpart on the right. The right parieto-occipital and the left middle frontal cerebral regions of the cast are thus asymmetrically reduced in fullness.

In norma lateralis view, Plates 12 and 13, while the low contour of the prebregmatic outline is clearly evident, its most striking departure from the canons of proportions as set by a modern standard is to be seen on the cast in its temporal regions. The notch between the temporal lobe and the cerebellar part of the cast is unusually wide and roomy, while from its prominent base, beginning at the level of the apex of the notch, the lobe itself tapers both forwards and inwards to the conical temporal pole in a manner not heretofore fully observed among the hominids. Except for a small strip along its uppermost boundary the whole lateral surface of the temporal lobe is inclined inwards. As a result of this curious under-development of the inferior temporal region, the well-developed and prominent pyriform area of the cast is widely exposed in norma lateralis. This exposure is to an extent greater than that of the corresponding region either in the endocranial cast of the Rhodesian skull or in the very primitively formed modern brain described by Elliot Smith in his "Essays on the Evolution of Man" (1927).

The superior temporal regions on each side in an area just below the parieto-squamous suture, between the sites where the latter is crossed by the main ramus lambdicus and the anomalous small ramus astericus of the meningeal arteries, constitutes the most salient lateral prominences of the cerebrum. On

either side the areas in question each constitute quite well-circumscribed bosses which are apparently homologous with the correspondingly prominent posterior temporal swellings of the Piltdown and Rhodesian endocranial casts to which Elliot Smith (1913) has repeatedly drawn special attention.

The form and relations of the parts about the roomy Sylvian depression are well displayed on the left side of the cast. In front of the depression the hinder part of the lower margin of the inferior frontal gyrus forms a large and prominent protuberance, which, on account of the peculiar form of the temporal lobe, is widely separated from the latter. Behind the inferior frontal protuberance lies a depressed area, somewhat triangular in outline. From this arrangement of the parts about the Sylvian depression there can remain no doubt that these may directly be homologised with the frontal and the fronto-parietal insular operculæ of which the frontal is particularly well developed. The arrangement and the development of the parts about the Sylvian depression provide good grounds for the supposition that the anterior insular region in *Simanthropus* was not fully covered by its operculæ. It is a matter of no little importance to add that the left frontal insular operculum is much larger and more prominent than its counterpart on the right.

One other point should be noted before concluding this general description of the cerebrum and passing on to a more detailed consideration of its lobes. The left middle frontal and the right parieto-occipital areas of the cast have been described as somewhat flattened and less prominent than their corresponding contralateral parts. It should be added, however, that the right inferior parietal region over a small area immediately above the temporo-cerebellar notch exhibits a prominent localised swelling of which a counterpart on the left is hardly to be recognised.

Juga Cerebralia and Impressiones Digitatæ.

Occipital Area.—Upon its cerebral occipital surface a number of faintly marked ridges and depressions may be distinguished on the endocranial cast and their form and extent may also be studied to advantage upon the endocranial surface of the separate cast of the occipital bone itself, which has earlier been described (*loc. cit. supra*). These markings may be seen as indistinct shadows in the photograph illustrated in Plate 15, but their relations and extent are indicated diagrammatically in fig. 6.

In the latter figure it becomes clear that the characteristic outline of a lunate sulcus is preserved on the left, which is the more prominent of the two

occipital areas. On the right side a less clear sulcal pattern is revealed, the lower fissure of which possibly represents the so-called lateral calcarine sulcus.

Temporal Area.—In a lateral view of the cast the position of the superior temporal sulcus is indicated quite distinctly in its anterior part on both right and left sides. On the right side the anterior part of the middle temporal sulcus may also be distinguished, but on the left its course is masked by the anomalous ramus astericus meningeal vascular groove. On both sides the small depression on the cast marked G in the figures represents the endocranial eminence caused by the external depression of the glenoid fossa. In the left norma lateralis view of the cast the prominent pyriform temporal area may be seen which in the norma basalis view is shown to be bounded laterally by a depression corresponding to the inferior temporal sulcus, figs. 2, 3 and 4.

Frontal Areas.—The left orbital frontal surface is well preserved and bears impressions upon its surface revealing much of the sulcal pattern of this region. This pattern may also be studied to advantage on the separate cast earlier prepared of the left fronto-sphenoidal part of the skull wall. In the norma basalis view in fig. 2 the lateral and medial extremities of the irregularly U-shaped orbital sulcus are indicated, the intermediate portion of this furrow being hidden by the projecting temporal pole. Rostral and medial to the orbital sulcus numerous other irregular sulcal markings are to be distinguished. Identification of the groove here homologised with the orbital sulcus is based upon the relation of its caudo-lateral portion which extends as a limiting sulcus between the orbital and frontal insular operculæ.

The supero-lateral surface of the frontal region is marked by a series of grooves which may be homologised with little fear of error as the superior and inferior series of frontal sulci. Immediately in front of the lower end of the linear elevation marking the coronal suture a pre-central depression begins which extends upwards on both sides from this origin in a manner somewhat similar to the course of the homologous depression on the cast of *Pithecanthropus* (Elliot Smith, 1927). It is to be noted further that the pre-central depression in *Sinanthropus* occupies a more rostral position above than at its beginning behind and above the frontal operculum.

Parietal Area.—Though numerous irregular rounded ridges and depressions are evident over the parietal areas of the cast it is difficult to come to relatively certain conclusions as to their identity and for this reason no detailed description of them will be attempted here. It is worthy of note, however, that the parietal is quite definitely demarcated from the frontal region proper by the well-marked pre-central boundary depression.

Summary and Conclusion.

It has been shown that the cranial capacity of this adolescent *Sinanthropus* skull attained a volume of approximately 960 c.c., a figure which falls well within the lower range of variability of measurements of this character normally to be observed among the adults of not a few modern human races. In view of the known ability of *Sinanthropus* in the usage of fire and in the manufacture of crude stone artifacts a volume of this magnitude is no less than was to be expected in an adolescent representative of the genus.

The grooves caused in the recent state by the major endocranial vascular sinuses are well marked on the cast. The superior longitudinal sinus groove becomes continuous with that representing the right lateral sinus, the relations of these vascular channels in *Sinanthropus* being thus similar to those most frequently encountered in modern man. The pattern of the vascular grooves of the middle meningeal artery conforms essentially on both sides of the cast with Giuffrida-Ruggeri's *Type IIa*, while displaying in minor respects some deviations from the latter.

From a study of the configuration of the cast over its cerebellar region it becomes evident that the right cerebellar hemisphere fossa is the more deeply excavated and roomy of the two. Since the cerebellum is an organ concerned chiefly in the elaboration of homolateral tonic, sthenic and static impulses of motor control, its manifest asymmetry in favour of the right side affords evidence indicative of the functional dominance of the musculature of that side of the body in *Sinanthropus*.

On the whole the cerebral region of the cast exhibits a remarkable degree of bilateral symmetry when compared with the marked asymmetries of the hemispheres so commonly characterising the brain of modern man. It is considered that among the latter this character is a specialised one. If this be so the general symmetry or rather the lack of marked asymmetry of the cerebral hemispheres in *Sinanthropus* may be regarded as being evidence of the retention by this form of a primitive character.

Notwithstanding their superficial symmetry, a closer examination of the cerebral regions of the cast makes it clearly evident that with but minor exceptions the left hemisphere in *Sinanthropus* exhibits an undoubted superiority in bulk over the right. This is especially evident in the parieto-occipital and inferior frontal regions of the cast. Since the cerebrum is primarily a suprasegmental organ concerned in contralateral afferent and efferent nervous control, its asymmetry in favour of the left side affords evidence of a con-

firmatory nature to that already adduced, of the functional dominance of the somatic organs of the right side of the body in *Sinanthropus*.

A consideration of the unique morphology of the parts about the Sylvian depression leads to the conclusion that in *Sinanthropus*, owing to the unequal and peculiar development of the four cerebral operculæ, its anterior insular region was most probably exposed.

The extraordinarily marked development of the opercular portion of the inferior frontal region on the left side in *Sinanthropus* is a matter of particular interest in view of the known location in this region of the modern brain of the motor speech centre. Thus in view of this peculiar development of Broca's convolution, it is considered probable that the mechanism of articulate speech was elaborated in *Sinanthropus*.

The interest in this mark of cerebral superiority is increased when it is recalled that, notwithstanding the quite anthropoid configuration of the tooth-bearing region of the *Sinanthropus* mandible (1931), the latter supported a dental battery of most generalised and progressive hominid type and its movements in articulation with the skull were essentially similar to those obtaining in modern man. Regardless of the archaic morphology of their jaws, the teeth themselves in *Sinanthropus*, *Eoanthropus* and *Palæoanthropus* are similarly generalised and distinctly progressive in type, while of those of *Pithecanthropus* this cannot be said. Unfortunately the inferior frontal region is not wholly preserved on the endocranial cast of *Pithecanthropus* so that a fair comparison of its development in that form and in *Sinanthropus* is not possible, nor is it possible to compare the latter form with *Eoanthropus* in this respect. The speculation may, however, be permitted here that there is more than a fortuitous relationship between the morphological characters of the teeth of early hominids and their cerebral development. It is not impossible that a direct relationship exists between the development in hominids of the mechanism of articulate speech and the character of their dental morphology.

The convolutional pattern of the cerebrum so far as it may be determined from the cast with any degree of certainty is such as might be expected to obtain in any primitive human brain. Attention has been directed to the presence on the cast of a well-marked pre-central depression and to the fact that its upper end is most rostrally placed. The obliquity in the course of this depression in relation to the line of the coronal suture is probably of significance as an indication of a vertical placement of the sulcus centralis since the depression itself is but the expression of the anterior boundary of a well-expanded pre-central cortex.

In conclusion, it may be said that the endocranial cast of *Sinanthropus* presents both in its general form and in the details of its morphology an interesting blend of archaic, generalised and progressive characters quite in keeping with what is already known about the external skull morphology of this form. From the evidence furnished by a detailed study of this cast but little room for doubt remains that this early hominid was provided with the essential nervous mechanism for the production of articulate speech. Finally, concerning the probable significance of the cerebellar and cerebral asymmetries noted above it is of interest to add that in their recent memoir Teilhard and Pei have shown that not a few of the Choukoutien artifacts "seem to have been handled with the right hand" (Teilhard de Chardin and Pei, 1932).

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577 . 16 D : 541 . 141

On the Surface Potentials of Unimolecular Films of Ergosterol.—The Photochemical Formation of Vitamin D.

By RUSSEL J. FOSBINDER, Cancer Research Laboratories, Graduate School of Medicine, University of Pennsylvania.

(Communicated by E. K. Rideal, F.R.S.—Received June 14, 1932.—Revised October 21, 1932).

Abstract.

The surface potentials and force-area curves of films of ergosterol and its irradiation products on the surface of a citric acid-NaOH buffer have been examined.

From a study of the behaviour of ΔV and μ on irradiation, a suggested mechanism for the photochemical formation of vitamin D is proposed.

The full paper is published in 'Proc. Roy. Soc.,' A., vol. 139, p. 93 (1933).

541 . 141 : 547 . 869 . 2 : 576 . 809 . 6

The Photodynamic Action of Methylene Blue on Bacteriophage.

By J. R. PERDRAU and CHARLES TODD, F.R.S.

(From the National Institute for Medical Research, Hampstead.)

(Received November 8, 1932.)

In the course of some work on the trypsin susceptibility of two races of staphylococcus bacteriophage, Schultz and Krueger (1928) made the observation that these two phages were remarkably sensitive to the action of methylene blue—being completely inactivated within 6 to 12 hours by concentrations of the dye as low as 0.002 per cent. So far as their tests went, the susceptibility to methylene blue appeared to be peculiar to the two races of staphylococcus bacteriophage, as eight phages for other organisms were entirely unaffected by the dye. Moreover, of a considerable number of dyes used, methylene blue was the only one showing this action. Clifton and Lawler (1930) and Burnet and McKie (1930) repeated these experiments with methylene blue and other dyes on several strains of bacteriophage, but they were then unaware of the role played by light in these reactions.

A new aspect of the mechanism of the reaction was opened up by Clifton (1931), who found that no inactivation took place when the dye and the phage were mixed, incubated and tested in the dark; but that an exposure of 5 minutes, or even less, to indirect sunlight before incubation resulted in complete inactivation of the bacteriophage. He also showed that the presence of oxygen was necessary for the process, as inactivation did not occur *in vacuo* or in an atmosphere of nitrogen, and was inhibited by reducing agents such as cysteine hydrochloride. He therefore concluded that the inactivation of the staphylococcus phage by methylene blue is due to an oxidation of the phage by photosensitized methylene blue in the presence of oxygen.

We have repeated and extended the experiments of Clifton with the same strain of bacteriophage which he used, namely, the staphylococcal strain of Krueger, kindly given us by Dr. C. H. Andrewes. We have also made a number of observations on two phages which we have found to be very sensitive to the photodynamic action of methylene blue. These are a dysentery Y bacteriophage and a second staphylococcal strain (referred to as "coccus S.F." in this paper). For the latter we are indebted to Dr. F. M. Burnet. These

three phages behave quite specifically on their respective organisms and no trace of interaction has been detected between them. Owing to the absence of technical details in Clifton's short paper, these have been included here, as they form the basis of our subsequent observations on the photodynamic action of methylene blue on viruses.

Methods.

After some tentative experiments in which the staphylococcus bacteriophage was found to be extremely sensitive to the combined effect of light and methylene blue, a uniform technique was adopted—quite arbitrarily—in order that the results should be comparable.

The exposures were made in a photographic dark room, a 100-c.p. pointolite lamp being used as the source of light. This was fixed at a distance of 20 cm. above the bench. The intensity of the light showed some variation owing to fluctuations in the voltage of the private supply of the Institute, but was sufficiently constant for practical purposes.

The volume of fluid exposed was such as to give a depth of approximately 2 mm. in a Petri dish, 10 c.c. being used for dishes of 8 cm. diameter and from 2.0 to 2.5 c.c. for dishes of 4 cm. For the exposures the dishes were placed on white paper and their lids were raised from a half to 1 cm. in order to prevent condensation of water vapour on their lower surfaces. The methylene blue used was Grüber's "Methylenblau, f.Bac.Koch."

In carrying out the tests equal volumes of a broth filtrate of the bacteriophage (which in the case of the staph. K. phage was usually active at a dilution of 10^{-8}) and of a solution of methylene blue in saline were thoroughly mixed by pouring several times from tube to tube in the dark or in a subdued indirect light. During the exposure samples were taken at intervals, either with a platinum loop or with a capillary pipette of standard bore, into tubes containing 5 c.c. of broth and the latter were immediately transferred to a light-proof box. In the earlier experiments these tubes were simply inoculated with the corresponding organism and then incubated, but this method had the disadvantage that it merely showed the presence or absence of the phage and gave no indication of the amount present at any particular time. It was therefore superseded by a plating method in which a drop of the mixture, or of a suitable dilution of this, was mixed with a drop of a 24-hour old broth culture of the corresponding organism and then rubbed out on a 1 per cent. agar plate. The number of plaques was counted after incubation for 24 hours at 37° C.

While the figures obtained in this way, as shown by Krueger (1930), do not accurately represent the number of phage particles present, they give a good indication of the rate at which the inactivation is proceeding, and enable the end-point to be determined sufficiently sharply for the purpose of the experiments.

Photodynamic Action of Methylene Blue on Staphylococcus Bacteriophage.

We had no difficulty in confirming the principal claims of Clifton in regard to the inactivation of the staphylococcus bacteriophage. Concentrations of the dye from 1 in 10,000 to 1 in 1,000,000 were used. The results were very consistent, the small variations noted being clearly due to a slightly fluctuating illumination. The figures in Table I are averages of the figures obtained in a number of experiments carried out under the same conditions.

Table I.—Effect of the Concentration of Methylene Blue on the Inactivation of the Phage.

Exposure.	Concentrations of methylene blue.		
	1 in 10,000.	1 in 100,000.	1 in 1,000,000.
0 minutes.	U	U	U
2 "	2000 plaques	300 plaques	U
4 "	500 "	5 "	U
8 "	100 "	0 "	U
16 "	10 "	0 "	U
32 "	0 "	0 "	58 plaques

U = uncountable number of plaques.

If allowance is made for the dilution, the figures in Table I represent in each case the average number of plaques resulting from the plating of approximately 1/8000 c.c. of the irradiated mixture.

It will be seen that this particular strain of bacteriophage (*Staphylococcus kruegeri*) is extremely sensitive to the combined action of methylene blue and light and also that a concentration of the dye of 1 in 10,000 is less effective than one of 1 in 100,000. This result, which is probably to be attributed to the screening effect of the upper layers of the mixture, will be referred to later.

In our earlier experiments, and before we were aware of the remarkable sensitiveness of the phage to light in the presence of methylene blue, exposures to the pointolite lamp were made on a bench at the back of the laboratory in the very variable diffuse daylight of a London winter, and irregular results gave a good deal of trouble. These were overcome by the use of a

photographic dark room, but even here care had to be exercised in taking the samples and in preparing the dilutions previous to plating, as the ordinary red light of the dark room was found to be by no means inactive.

In order to gain an idea of the minimum exposure which would show an appreciable effect on the phage an experiment was carried out in which all manipulations, apart from the actual exposure, were made in absolute darkness.

One cubic centimetre of the staphylococcus phage was thoroughly mixed with 1 c.c. of a 1 in 50,000 solution of methylene blue in saline by pouring backwards and forwards from tube to tube and the mixture then poured into a small Petri dish of 4 cm. diameter, placed at a distance of 20 cm. from the pointolite lamp, and covered with a light-proof cover. The lamp was turned on, the cover removed and the desired exposure made, after which the lamp was turned off. The first dilution, which had to be made in complete darkness, was effected by pouring the contents of the dish into a Winchester quart bottle containing 1800 c.c. of tap water and fitted with a funnel, the dish being then washed out over the funnel with a further 200 c.c. of water—making a total of 2 litres (*i.e.*, a dilution of 1 in 1000 of the original fluid). In a dim light 1 c.c. of the contents of the Winchester was now added to 9 c.c. of water in a test tube, making a dilution of 1 in 10,000 of the original fluid. As this dilution represented a concentration of only 1 part of methylene blue in 10^9 —at which strength the dye is quite inactive—further dilutions could safely be carried out in diffused daylight.

A series of exposures were made in this way and the dilutions plated on 1 per cent. agar.

In Table II is a summary of the results.

Table II.

Exposure.	Number of plaques.
0	1524
10 seconds	985
1 minute	442
2 minutes	5
4 „	0

The figures represent the number of plaques given by one drop of a 1 in 10 dilution of the contents of the Winchester. From Table II it will be seen that the process of inactivation begins from the moment that the mixture is exposed to light.

Action of other Dyes.—Comparatively few experiments with other dyes have been carried out in the course of this investigation. Clifton and Lawler have

claimed a similar but weaker action on the staphylococcal phage for toluidine blue and our own limited experience confirms this claim.

The degree of fluorescence possessed by the dye is apparently not a measure of its photodynamic activity on a susceptible phage, since highly fluorescent dyes such as eosin and pyronin were found to be quite inactive even after prolonged exposures.

Protection of the Phage by the Presence of Living Organisms.

It is a general experience that, if living bacteria are added to a filtrate of the corresponding phage, the phage is somewhat rapidly removed from the fluid and fixed by the susceptible organisms. As the nature of this union is unknown we were interested to see if the staphylococcus phage, which in its free state is so highly sensitive to the photodynamic action of methylene blue, is still susceptible to the dye after its union with the staphylococcus.

For this purpose 5 c.c. of the staphylococcus phage were mixed with 8 drops of a 24-hours' broth culture of the staphylococcus and, after contact for 10 minutes at room temperature, 5 c.c. of a 1 in 5000 solution of methylene blue in saline were added and the mixture exposed in the usual way to the light of the pointolite lamp. Drop samples were taken at intervals and suitable dilutions of these mixed with the staphylococcus and plated. The results are given in Table III.

Table III.—Effect of the Presence of Living Staphylococci on the Photodynamic Inactivation of Staphylococcus Phage by Methylene Blue.

Time of exposure.	Number of plaques on agar.
minutes	
0	U
5	2100
10	410
20	280
40	114
120	20

Thus, even after 2 hours' exposure, the phage was not completely inactivated, so that the presence of living staphylococci had in some way protected it from the photodynamic action of the dye. This experiment was repeated a number of times with slight modifications and protection was always obtained, provided that the irradiation was not excessive. If the order in which the phage and the dye reach the bacterial cell is reversed, that is to say, if the methylene blue and the culture are allowed to be in contact for 10 minutes

before the addition of the phage, which is also allowed to be in contact for 10 minutes before irradiation, the result is the same as that given in the table.

In order to determine whether dead organisms protect as efficiently as living ones, a similar experiment was carried out with the following bacterial suspensions :—

- (a) Staphylococci killed by heat (65° C. for 30 minutes).
- (b) Staphylococci killed by exposure to chloroform vapour.
- (c) Living staphylococci.

In this case the optimum concentration (1 in 100,000) of methylene blue was employed and in order to favour the union of the organisms with the phage the mixture was kept for 10 minutes at 37° C., instead of at room temperature, so as to give a better chance of union ; the results are given in Table IV.

Table IV.—Effect of the Presence of Living and of Dead Staphylococci on the Photodynamic Inactivation of Staphylococcus Phage by Methylene Blue.

Time of exposure.	Staphylococci killed by heat.	Staphylococci killed by chloroform.	Living staphylococci.
0 minutes	U	U	U
5 "	86 plaques	309 plaques	U
10 "	0 "	0 "	U
20 "	0 "	0 "	U
60 "	0 "	0 "	U

U = uncountable number of plaques.

Protection of the bacteriophage against the photodynamic action of methylene blue is therefore a property of the living and not of the dead organism.

Time of Contact.—It was thought that the protection phenomenon might be used for the purpose of determining how quickly the union of the phage and its organism is brought about. For this purpose four drops of a young broth culture of the appropriate organism were added to 1 c.c. of a filtrate of the phage, and after varying periods of contact at room temperature, methylene blue was added and the mixture immediately irradiated. For these experiments the pointolite lamp was unsuitable as with its comparatively feeble illumination protection of the phage was observed over a period of several hours, during which either further union of the phage with the organism or an actual multiplication of the phage may have been taking place. A more powerful source of light was therefore desirable and sunlight was resorted to.

Contact between the phage and the organism was allowed to proceed at room temperature for periods of 1 to 16 minutes and samples of the mixture were tested for the presence of the phage at intervals during the exposure.

The result of an experiment made in London at mid-day, on a sunny day in the month of October, is given in Table V, which shows that the protection afforded by the living cells increases progressively with the time of contact of the organism with the phage.

Table V.—Showing the Influence of the Time of Contact between the Phage and the Living Organism on the Time required for Inactivation by subsequent Irradiation (Sunlight).

Time of contact of phage and organism before irradiation.	Time of irradiation required to inactivate.
minutes	minutes
0	1
1	1
2	2
4	4
8	30
16	60

When contact between the phage and the organism took place at 37° C. a much higher degree of protection was obtained than at 20° C.; the time required for inactivation of the phage at 37° C. being many times that required at room temperature.

Cross Protection with two other Susceptible Phages.

Two other phages which were found to be susceptible to the photodynamic action of methylene blue were used, with their respective organisms, in an attempt to determine whether cross protection was afforded by the presence of living heterologous organisms. These phages were those for a strain of *B. dysenteriae* Y, and for a white staphylococcus (Coccus S.F.). In the presence of the optimal concentration of methylene blue needed for the inactivation of the staphylococcus (Krueger) phage (1 in 100,000) the dysentery phage was completely inactivated in 4 minutes and the Coccus S.F. phage in 8 minutes.

The results of tests of the cross protection of the three strains of phage and the corresponding organisms are given in Table VI.

Table VI.—Summary of Experiments on the Protection of Three Phages from the Photodynamic Action of Methylene Blue by Living Organisms.

	Coccus S.F. phage.	Staphylococcus K. phage.	Dysentery Y phage.
Coccus S.F.	P	P	P
Staphylococcus K.	0	P	P
Dysentery Y	0	0	P

0 = not protected; P = protected.

These results show that—

- (a) in each case the phage is protected by its own organism ;
- (b) the dysentery phage is protected by all three organisms ;
- (c) coccus S.F. protects all three phages ;
- (d) no protection results from the other combinations.

It is thus clear that the property of protecting the phage is not limited to the homologous organism, but may be shown by other bacteria.

Necessity for the presence of Free Oxygen.—It has been known since the work of Ledoux-Lebard (1902) and Jodlbauer and Tappeiner (1905) that the presence of free oxygen was essential in photodynamic reactions. (Lifton (1931) noted a similar requirement for the inactivation of staphylococcus (Krueger) phage by methylene blue, and thereby demonstrated the photodynamic character of the reaction in contradistinction to the destructive effects of ultra-violet light, for which the presence of free oxygen is not necessary (Blum, 1932).

In our first experiments mixtures of the staphylococcus phage with equal volumes of solutions of methylene blue were prepared and placed (in the dark) in two small Petri dishes on the bottom of a MacIntosh and Fildes anaerobic jar, the sides of which were covered with black paper to exclude light. The concentrations of methylene blue in these two dishes were 1 in 10,000 and 1 in 100,000 respectively. The jar was then de-oxygenated and kept in a dark cupboard overnight. Next morning the de-oxygenation was repeated and the jar was placed on a glass plate at 20 cm. above the pointolite lamp and irradiated from below. After an exposure of an hour the jar was opened and samples taken from the two mixtures, both of which showed the presence of the phage. The two fluids were then oxygenated by passing a stream of oxygen and the jar again exposed to light in the same manner for 20 minutes. Samples now taken were found to be quite free from the phage.

The experiment was repeated a number of times with identical results, showing that the photodynamic action of the dye does not take place in an atmosphere of nitrogen.

When the irradiation is carried out *in vacuo* the dye is similarly inactive. For this purpose a U-tube furnished with a glass tap in the horizontal portion connecting the two limbs was used, the phage being placed in one limb and the methylene blue solution in the other. The two limbs were closed by rubber stoppers fitted with glass tubes which were connected by rubber tubing with a vacuum pump. After washing out repeatedly with hydrogen the limbs were finally evacuated, the connecting tap opened, and the fluids in the two limbs mixed by tilting the apparatus backwards and forwards. The apparatus was

now placed on the bench in front of a window and exposed to diffused daylight for 50 minutes after which it was taken into the dark room, the tap was closed and one limb clipped off as a control—by clamping the rubber tubing—and the other opened and sample drops taken out and plated. The plates showed an uncountable number of plaques. The fluid in the opened limb was then oxygenated by a stream of air and the apparatus exposed to daylight for a further 50 minutes. At the expiration of this time the presence of phage could not be detected in the oxygenated limb, while the amount of phage in the control exhausted limb, which had now been exposed to daylight for 100 minutes, showed no appreciable diminution. These results are entirely in accordance with the observations of Clifton (1931).

Attempted Reactivation of the Inactivated Phage.—Perdrau (1931) found that in a certain number of cases, broth filtrates of herpes could be inactivated by molecular oxygen and subsequently reactivated by a process of reduction. The phenomenon was by no means a constant one, since it was observed in only 4 out of 13 filtrates; but the experiment could be repeated on filtrates which had once been proved vulnerable to oxygen. When the work was done the possible action of light was not considered and the experiments were made at different times and under widely different degrees of illumination, sometimes in diffused daylight and at other times by artificial light. It seemed not impossible that the inactivation of the virus might have been due to the action of some photodynamically active substance present, probably in very small amount, in the filtrates, and the inconstancy of the results to variations either in the amount of this substance present or in the intensity of the illumination during the process of oxygenation.

Whatever the mechanism by which the inactivation of herpes virus was brought about, the interesting point was that it was apparently reversible by subsequent reduction. In view of these results a number of attempts were made to effect a similar reactivation of the staphylococcus phage after its inactivation by the photodynamic action of methylene blue, by treatment with hydrogen in the presence of colloidal palladium. The reduction was carried out under various conditions—in the dark and under exposure to light, for various times, etc.—but in all cases with uniformly negative results.

Influence of the Nature of the Light used for Inactivation.

As stated above, the source of light employed in these experiments was that of a 100-c.p. pointolite lamp. A certain number of tests were, however, carried out in which the light was filtered through colour screens.

The exposures were made as usual at a distance of 20 cm. from the lamp, but various screens were interposed between the lamp and the mixture under irradiation—suitable arrangements being made to prevent the access of unfiltered light.

A mixture of staphylococcus phage with a solution of methylene blue, containing the optimal concentration (1 in 100,000) of the dye was exposed in this way to red light. For this purpose a combination of Wratten A and F screens was used, and samples of the mixture were examined at intervals. Almost complete inactivation of the phage was obtained in 8 minutes, and complete inactivation in 16 minutes. Further experiments were made with green light. Here a mercury arc was used as the source of light, which was filtered through a combination of Wratten "B No. 58" and Ilford "Mercury green" screens. The counts obtained at the beginning and at the end of the experiment—half an hour's exposure—were 202 and 176 respectively. These figures are within the limits of experimental error and they suggest that a green light is a safe one in which to carry out the manipulations, even though, as in this experiment, a yellow band as well as a green one can be seen on spectroscopic examination.

In order to see whether the inactivation of the phage obeys the absorption law of Grotthuss-Draper—that only light which is absorbed is possessed of any chemical activity—screens of methylene blue in various concentrations were interposed between the pointolite lamp and a mixture of the phage with the most effective concentration of the dye (1 in 100,000). It was found that a screen consisting of a layer of 1 in 10,000 of methylene blue in a cell 1 cm. deep was sufficient to stop all inactivation of the phage even after an exposure of 30 minutes.

An explanation is here afforded of the previously recorded observation that, without the interposition of any screens, a mixture of the phage with 1 in 10,000 of the dye is less rapidly inactivated than one containing 1 in 100,000 of the dye, the upper layers of the mixture in the case of the stronger concentration probably having a screening effect on the lower ones.

As the main absorption band of methylene blue is in the red, we see why a red screen does not prevent the inactivation of the phage while a green one does. We have not gone into the question of the exact wave-length of the active rays, as this could only be determined by the use of spectrally pure light of known wave-length and intensity.

A test of the shorter infra-red rays was also made. The light of the pointolite lamp, after traversing a solution of iodine in carbon disulphide of such strength as just to stop the passage of visible light rays, appeared to be quite inactive.

As a number of the ultramicroscopic viruses have been found to resemble the phage in their sensitivity to the photodynamic action of methylene blue and form the subject of a separate communication, any discussion is deferred until these have been dealt with.

Summary.

(1) Evidence is brought forward confirming Clifton's observations that the staphylococcus (Krueger) phage is highly sensitive to the photodynamic action of methylene blue and that this inactivation of the phage does not take place in the absence of oxygen.

(2) Another staphylococcal phage, as well as one for *B. dysenteriae* Y, were found to be equally susceptible.

(3) The optimal concentration of the dye is about 1 part in 100,000—stronger solutions being less effective probably owing to absorption of the light by the upper layers of the fluid.

(4) The inactivating action of visible light is confined to certain wave-lengths and can be abolished by the interposition of a screen of methylene blue.

(5) All attempts at reactivating the inactivated phage by reduction methods were unsuccessful.

(6) While the free phage is highly sensitive to the photodynamic action of methylene blue, it becomes very much more resistant after union with the corresponding living bacterium.

(7) This "protection" of the phage is only exercised by living bacteria, killed organisms being without effect.

(8) Protection becomes progressively greater as the time of contact between the phage and the living organisms, previous to irradiation, is increased, and is brought about much more rapidly at 37° C. than at 20° C.

(9) The protection is not a strictly specific phenomenon, as it can be brought about by certain heterologous bacteria.

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The Photodynamic Action of Methylene Blue on Certain Viruses.

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In a previous communication (Perdrau and Todd, 1933), dealing with the photodynamic action of methylene blue on bacteriophage, we have recorded the results of experiments confirming the observations of Clifton (1931), both as regards the part played by visible light in bringing about the reaction and also as regards the necessity for the presence of free oxygen. In the course of these experiments it was found that while the free phage is highly sensitive to the photodynamic action of methylene blue, it becomes very much more resistant to the dye in the presence of the corresponding living bacterium. This protection of the phage is only exercised by living bacteria, killed organisms being without effect. It is, moreover, not a strictly specific phenomenon, as protection is in some cases brought about by bacteria other than those for which the phage has been prepared.

In view of the many similarities of behaviour shown by the bacteriophage and the ultramicroscopic viruses, it seemed not improbable that some of the latter might be susceptible to the photodynamic action of methylene blue, and tests were accordingly made with such viruses as we had at our disposal.

The methods and the dye employed were the same as those used in the case of the bacteriophage, the source of light being a 100-c.p. pointolite lamp at a distance of 20 cm. from the fluid under irradiation, which was contained in a Petri dish lying on a sheet of white paper, and was covered by a glass lid. After varying periods of irradiation the fluid was tested on animals. The results of a large number of experiments (also shown in tabular form—Table I) are summarised below.

Behaviour of the various Viruses to the Photodynamic Action of Methylene Blue.

The filtrates used in the following experiments were from Pasteur-Chamberland candles or collodion membranes (Elford), any exceptions being noted in the text.

Herpes.—The strain (E.L.1) was originally obtained by one of us (J. R. P.) from the brain of a fatal case of epidemic encephalitis. Broth filtrates of infective brain as well as suspensions of old glycerinated brain were used. A broth filtrate (active at a dose of 0·2 c.c. of a dilution of 1/1000) was inactivated in 10 minutes at concentrations of the dye of 1/10,000 and 1/100,000. A 5 per cent. suspension of glycerinated infective brain ground up in saline was inactivated in the same time. The results obtained were quite regular.

Vaccinia.—Neurovaccine (Levaditi). Material used as for herpes. A broth filtrate (active at 1/10,000) with concentrations of the dye of 1/10,000 and 1/100,000 gave, after irradiation, no reaction in the skin of the rabbit in

Table I.—Minimum Time in Minutes for Inactivation of Cell-free Infective Material.

	Concentration of dye.			Remarks.
	1/10,000.	1/100,000.	1/1,000,000.	
Fowl plague	—	5	—	Blood plasma filtrate active at 1/100,000 c.c. Test dose: 0·2 c.c. of mixture intramuscularly in fowls.
Louping-ill	5	5	—	Broth filtrate active at 1/1,000,000 c.c. Test dose: one drop of mixture cerebrally in mice.
Herpes	10	10	30	Broth filtrate active at 1/10,000 c.c. Test dose: 0·2 c.c. of mixture cerebrally in rabbits.
Vaccinia . .	10*	10*	—	Broth filtrate active at 1/100,000 c.c. Test dose: 0·2 c.c. of mixture intradermally in rabbits.
Canine distemper	—	10	—	Centrifuged saline suspension of spleen active at 1/1,000,000 c.c. Test dose: 1 c.c. of mixture subcutaneously in ferrets.
Borna disease	—	20	—	Broth filtrate active at 1/10,000 c.c. Test dose: 0·2 c.c. of mixture cerebrally in rabbits.
Fujinami tumour	—	30 (or less)	—	Sand and paper pulp filtrate of duck tumour suspension in saline. Test dose: 1 c.c. of mixture intramuscularly in chicks.
Infectious ectromelia	†Results irregular			Broth filtrate active at 1/100,000 c.c. Test dose: 0·2 c.c. of mixture intraperitoneally in mice.
Foot-and-mouth disease	†Results irregular			Broth or phosphate saline filtrates. Test dose: one drop of mixture in pads of guinea-pigs.

* Traces of virus occasionally survived for 20 minutes when test dose was 10 c.c. of mixture subcutaneously.

† Since the above was written inactivation of these two viruses has been obtained with a more powerful lamp (300 c.p.).

doses of 0·2 c.c. of the mixture (*i.e.*, 0·1 c.c. of the original filtrate). But when larger volumes (10 c.c.) of the irradiated mixture were given subcutaneously a slight local reaction was occasionally obtained even after 20 minutes' exposure. Suspensions both of ground-up glycerinated brain and of calf lymph yielded similar results.

Louping-III.—The strain was one supplied to Mr. Galloway by the Moredun Institute, Edinburgh. Broth filtrates (active at 1/1,000,000) prepared from the brains of infected mice were found to be extremely susceptible, since inactivation was always complete in 5 minutes, with concentrations of the dye both of 1 in 10,000 and 1 in 100,000.

Fowl Plague.—Strain obtained from Professor Doerr. Filtrates of citrated virulent plasma, with or without added broth and active at 1/100,000 c.c., were completely inactivated in 5 minutes by methylene blue in a concentration of 1 in 100,000.

Borna Disease.—Strain *Zwick* (equine) passaged through rabbits by Mr. Galloway for a period of some years. Broth filtrates, prepared from the brains of infected rabbits and active at 1 in 10,000, were completely inactivated in 20, but not in 10 minutes; the concentration of the dye being 1 in 100,000.

Foot-and-Mouth Disease.—Strain G.F. (Type 0). Broth and saline filtrates of infective pads of guinea-pigs (active at 1 in 1,000,000) were supplied by Mr. Galloway and used in experiments involving 54 animals. The virus was found to be active even after 2 hours' irradiation, and it must therefore be regarded as comparatively resistant to the action of methylene blue, in concentrations of 1 in 10,000, 1 in 100,000 and 1 in 1,000,000.

Infectious Ectromelia.—Original strain *Marchal*. A broth filtrate of infective liver, active at 1 in 100,000, supplied by Capt. S. R. Douglas. The results (based on 32 experiments) were irregular and the susceptibility of the virus must be regarded as doubtful. The concentrations of the dye used were 1 in 10,000 and 1 in 100,000.

Canine Distemper.—Strain of Laidlaw and Dunkin. Infective ferret's spleen, kept frozen or in glycerin and active at 1/1,000,000 gm., was used. A centrifuged 1 per cent. suspension in saline was inactivated in 10 minutes by methylene blue in a concentration of 1 in 100,000.

Fujinami's Bird Tumour.—Sand and paper-pulp filtrate of a duck tumour supplied by Dr. C. H. Andrewes. A single sample was used, the concentration of the dye being 1 in 100,000, and the irradiation one of 30 minutes. The untreated material was inoculated into the right breast and then irradiated into the left breast of each of four chicks. Tumours resulted from the control inoculations only, in all four birds, so that this virus also is susceptible to the photodynamic action of methylene blue.

Controls.—The controls to these experiments as well as to others to be described subsequently, consisted of (1) a half dilution in saline of the virus suspension which was exposed to light for at least the longest period tested;

(2) a mixture of the virus and the dye kept in the dark for similar or longer periods. In neither case was inactivation of the virus ever observed.

Necessity for the Presence of Oxygen.

Experiments were carried out with the viruses of herpes and vaccinia in order to test the necessity for the presence of oxygen in these photodynamic inactivations. The apparatus and the technique used were the same as those previously described in the case of bacteriophage. With concentrations of the dye of 1/100,000, inactivation of both viruses took place in the limb of the U-tube which contained oxygen, but not in the other limb, from which oxygen was absent.

Protection of the Virus by Living Cells of Infected Animal.

It will be remembered that bacteriophage is very definitely, though not absolutely, protected against the inactivating effect of methylene blue and light when living bacterial cells, not necessarily homologous, are added to the phage filtrate a few minutes before the addition of the dye.

In order to test this phenomenon in the case of the viruses, an animal was killed at the height of the symptoms of the particular infection, and a virulent organ, usually the brain, was removed immediately and shaken up in a test-tube containing Ringer's solution. The resulting suspension was filtered through a double layer of gauze. Under the microscope the majority of the cells appeared to be free, though a few clumps were present.

To such a suspension of living infected cells methylene blue was added to yield a concentration of 1/10,000, and the mixture then exposed in the usual way to light for 30 or 60 minutes. The results are given in Table II. It was found that the viruses of herpes and of Borna disease were definitely protected against the photodynamic action of methylene blue by the living brain cells, the virus of fowl plague by living blood corpuscles or liver cells, and that of distemper by the living cells of the spleen.

Table II.—Protection by Living Cells of Infected Animal.

	Living cells.	Dead cells.	Organ or tissue.
Herpes	+	0	Brain.
Vaccinia	?	0	Brain and testis.
Louping-ill	0	0	Brain.
Borna disease	+	0	Brain.
Fowl plague.	+	0	Blood and liver.
Canine distemper	+	0	Spleen.

+ = survival of the virus. 0 = inactivation of the virus.

In the case of vaccinia only slight protection of the virus was obtained.

As a control to these experiments in which living cells were used, it was necessary to carry out similar experiments with dead cells. The difficulty of killing the cells, without inactivating the virus contained in them, was overcome by the use of suspensions made from infected brain which had been kept in 50 per cent. glycerin in the cold room for some weeks. These suspensions were highly infective. In all these experiments where dead cells were used a rapid inactivation took place.

Absence of Protection of the Virus by Living Normal Cells.

It was not found possible to replace the living cells of an infected animal by cells obtained from a normal animal and allowed to remain in contact with a saline or Ringer suspension of the virus. Here inactivation always took place.

The viruses and living normal cells used for these experiments were: herpes (brain, testis); vaccinia (testis, spleen, kidney, blood); fowl plague (blood).

Photodynamic Action of Methylene Blue in the Living Animal.

As the result of experience gained from the experiments recorded above, observations were made on the treatment of virus infections in the living animal. For this purpose only viruses capable of producing lesions on the surface of the body were used, in order that adequate exposure to light could be realized. Of the viruses studied those of vaccinia and of herpes were obvious choices, as they both produce characteristic skin and corneal lesions. Vaccine virus was used for both methods of inoculation, but in the case of herpes the corneal route only was employed, as the strain at our disposal did not produce satisfactory skin lesions owing to its marked neurotropism. The general nature of the experiments consisted in inoculating the virus into the skin or the cornea and, then at varying intervals, applying the dye locally or giving it intravenously.

Rabbits only, preferably adult animals, were used, white Himalayan for the skin inoculations, and both Himalayan and Dutch for corneal infection.

In the skin experiments virulent brain (neurovaccine) was rubbed into two long scarifications, one on either flank, and in the corneal experiments a small area not larger than 2 mm. square was scarified halfway between the centre of the pupil and the outer margin of the cornea, and infective brain rubbed

in. The methylene blue used in these experiments was "Methylene blue free from arsenic, B.D.H."

For local application the dye was used in a concentration in saline of 1 in 200 and for intravenous injection a 1 in 400 dilution in saline. Rabbits weighing from 1800 to 2000 gm. tolerated 20 c.c. of this concentration without showing any obvious toxic symptoms, and young rabbits rather less than the proportional dose of the dye relative to their weight. After treatment with the dye the rabbits were exposed on a balcony either to bright daylight or to direct sunlight, the experiments being carried out in summer. Both corneal scarification and the subsequent treatment were carried out under cocaine anaesthesia. For either type of inoculation both sides of the body were used, and in the case of local applications only one side was treated, the other side acting as a control.

The results are given in Table III.

Skin Scarification.—For the reasons already stated only vaccine virus was used. Treatment by intravenous injections showed that no "take" resulted if the dye was given up to 18 hours after inoculation of the virus, but that, when it was given 24 hours after inoculation, the development of the virus was not entirely prevented, since, of the animals treated in this way, two out of three showed slight reactions.

Corneal Inoculations.—Irrigation of the conjunctival sac with the dye was uniformly successful in preventing the onset of a keratitis up to 4 hours after inoculation with neurovaccine, but only partially so after 12 hours (two successes and one failure). Very similar results were obtained in the case of herpes.

Table III.—Summary of results of Treatment in the Living Animal.

Time before start of treatment.	Vaccinia.			Herpes.			
	Cornea — treated locally.			Skin—treated by intravenous injection.			
5 minutes	0	0		0		0	0
30 "		0		0		+	
2 hours		0		0		0	0
4 "		0		0		0	0
12 "	0	0	+	0		0	
18 "	+	+	+	0		0	+
24 "				0	± ±	+	*
48 "				+		+	

+ = take. 0 = no take. * control failed to take.

Intravenous injections of the dye were without effect after corneal infection with either virus, and also after intradermal inoculations of a broth filtrate of neurovaccine, and these attempts were not continued.

Discussion.

The experiments on bacteriophage which preceded those recorded in this paper, were not then discussed, as it was thought more profitable to accumulate parallel data on the inactivation of the viruses, in order to have more material for purposes of comparison.

Viruses as well as different strains of bacteriophage vary in their susceptibility to the photodynamic action of methylene blue. No correlation has been found between the degree of susceptibility of the viruses and their affinity for a particular tissue. For instance, although all the neurotropic viruses tested were found to be susceptible, 3 out of 5 of the non-neurotropic viruses were also found to be vulnerable, namely, those of fowl plague, canine distemper and the bird tumour of Fujinami. Similarly, one dysentery and two staphylococcal strains of bacteriophage were found to be susceptible to methylene blue, but a *B. coli* phage was resistant.

The composition of the fluid in which the phage or the virus is suspended seems to be immaterial, and broth and saline are interchangeable in this respect. The previous findings of Clifton on the necessity for the presence of oxygen in the photodynamic inactivation of a staphylococcal phage have been confirmed, and a similar requirement has been demonstrated in the case of the viruses of herpes and of vaccinia—the only ones so tested. That these reactions are essentially oxidations has been known since the work of Ledoux-Lebard (1902) and Jodlbauer and Tappeiner (1905). This question has been reviewed recently by Blum (1932) who came to the conclusion that “the formation of peroxides at some point in such reactions seems clearly demonstrated,” and that these photodynamic reactions are probably effected by the direct action of peroxides, since similar effects can be brought about by hydrogen peroxide. We have tested the action of hydrogen peroxide on the staphylococcal phage of Krueger and on the viruses of vaccinia and herpes. All three of these substances are inactivated by methylene blue and light in 10 minutes, but, although the two viruses can be inactivated in a few minutes by hydrogen peroxide (1/300), the phage is more resistant, since a contact of 1 hour is necessary for its complete inactivation. Had the photodynamic reactions been effected through the intervention of hydrogen peroxide one

would have expected the phage to be as susceptible to this reagent as to methylene blue and light. The possibility that an organic peroxide is formed in the course of the reaction, and is responsible for these inactivations, is also discussed by Blum.

It has been claimed that in certain photodynamic reactions a sensitizer which has been exposed to light is subsequently capable of performing its specific function in the dark. We have controlled our experiments by adding to a virus or phage suspension, in the dark, methylene blue which had been exposed in test-tubes at appropriate dilutions to bright daylight for several weeks, without obtaining any traces of inactivation. Herpes virus, for instance, was still unaffected after a week's contact in the dark with the previously irradiated dye.

The most interesting of our observations was that of the "protection" of the susceptible phage or virus by living bacterial or animal cells respectively. The nature of this protection is unknown, but it seems reasonable to attribute it to the penetration of the phage or virus into the living cell, in the interior of which the reduction-potential is such that the photodynamic inactivation, requiring as it does the presence of molecular oxygen and of unreduced methylene blue, cannot take place.

In view of our lack of exact knowledge of the oxidation-reduction potential conditions existing in the interior of living animal and vegetable cells, this view can at present only be regarded as a working hypothesis, but as such it appears to fit in well with the experimental results so far obtained. It might be thought that it is unnecessary to postulate an actual penetration of the cell, and that the phage or virus might be protected in a zone of high reduction potential existing in the immediate neighbourhood of the living cell. Against this view, however, there is the fact that susceptible viruses are not protected *in vitro* by the presence of normal living cells.

In the case of the phage the protection phenomenon is easy to demonstrate. A drop of culture of the corresponding organism is added to a filtrate of the phage before the addition of the dye and exposure to light. Instead of the phage being inactivated in a few minutes, it is still active after some hours of irradiation. According to the view suggested above the protection of the phage would be due to its having actually penetrated the living cells present in the mixture. This point is difficult to prove, and the entry of the phage into the cell, if it occurs, must take place with great rapidity. That such penetration does actually take place was, however, suggested by the observation that protection is progressively increased as the time of contact between

the phage and the cell is prolonged; moreover, for the same time of contact the protection afforded at 37° C. is very much greater than at 20° C.

It is extremely unlikely that in the protection experiments with bacteriophage any multiplication of the phage could have taken place in the short period of contact with the bacterial cells previous to irradiation. Parallel experiments with the viruses were carried out by adding virus preparations to suspensions of suitable living cells from a normal animal, but no protection was ever obtained even after long periods of contact. When, however, the suspensions of living cells were obtained from an infected animal the protection phenomenon was observed with certain viruses. It would appear, therefore, that infection of the cells by the viruses can only be effected *in vivo*, presumably by multiplication of the virus. Even here the behaviour of the several viruses was far from being uniform. The viruses of herpes, Borna disease and fowl plague were definitely protected, that of vaccinia only partially, and that of louping-ill not at all.

On the hypothesis suggested above, protection of the virus would be explained by the presence in the irradiated mixtures of infected cells, or portions of cells still in such a condition of physiological activity as to be capable of maintaining in their interior the necessary reduction potential. This view is supported by the fact that, when a similar experiment is made with infective tissues which have been kept for some months in the cold, with or without glycerin, and where the living virus is contained in dead cells, no such protection is observed.

There is one feature in connection with the protection of the phage by the living bacterial cell which deserves special mention. It was found that not only were all three of the susceptible phages studied protected by their homologous cells, but also that protection could be afforded by certain heterologous cells, even though the phages were strictly specific in their lytic activity. This lack of specificity in the protective action of the bacteria is difficult to explain, except on the assumption that the phages in question are able to penetrate heterologous bacteria in which they are unable to multiply. An attempt was made to obtain evidence of multiplication under such conditions, but without success.

As already stated the photodynamic effect of methylene blue on virus infections in the living animal could only be tested in the case of the viruses of vaccinia and herpes, owing to their ability to produce lesions on the surface of the body. It was found possible to arrest the development of these lesions provided that the initiation of treatment was not delayed too long after

inoculation of the virus. The comparatively short intervals (12 to 24 hours) which could be allowed to elapse between the inoculation of the virus and the start of treatment may quite possibly represent the time which these two viruses take to invade the cells of the body, and thereby to become protected against the action of the dye.

We are indebted to several of our colleagues for the supply of some of the infective material used in this investigation, and particularly to Mr. I. A. Galloway and to Dr. P. P. Laidlaw and Major G. W. Dunkin, who, in addition, carried out a number of the animal tests.

Summary.

(1) The viruses of vaccinia, herpes, fowl plague, louping-ill, Borna disease, Fujinami's tumour and canine distemper, as they exist in filtrates or other fluids devoid of living cells are highly sensitive to the photodynamic action of methylene blue—a concentration of 1 part in 100,000 inactivating each of these viruses within a few minutes under suitable illumination.

(2) The viruses of foot-and-mouth disease and of infectious ectromelia, on the other hand, are more resistant to the photodynamic action of the dye.

(3) The inactivation of the viruses by methylene blue and visible light appears to be an oxidative process, as in experiments with the viruses of herpes and vaccinia no inactivation took place in the absence of free oxygen.

(4) The viruses of herpes, Borna disease and fowl plague, as they exist in freshly prepared suspensions of *living* cells from infected animals, are very much more resistant to the photodynamic action of the dye than they are in cell-free filtrates. This "protection" of the viruses is only exercised by living cells—dead cells being without effect.

(5) No such "protection" by the living animal cell was observed in the case of the virus of louping-ill, and in that of the virus of vaccinia the amount of protection obtained was very small.

(6) The development of the specific lesions of vaccinia and herpes on the surface of the skin or cornea can be prevented by suitable treatment with methylene blue and light; but, to be effective, the treatment must take place within a period varying from 12 to 24 hours after inoculation.

[*Note added in proof January 12, 1933.*—Since this paper was submitted, our attention has been drawn to a communication by Herzberg ('Z. Bakt.,'

vol. 122, p. 231, 1931), who studied the photodynamic action of methylene blue on the virus of vaccinia, and obtained results very similar to ours.]

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The Kinetics of Hæmolysis in Colloidal Silicic Acid -Complement Systems.

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The hæmolytic systems obtained by adding complement to red cells sensitized with colloidal silicic acid were first described by Landsteiner and Jagie in 1904, and have excited considerable interest ever since, principally because of their apparent similarity to amboceptor-complement systems. No attempt, however, has been made to investigate their kinetics (a fact which is hardly surprising in view of their extraordinary complexity), and this paper is concerned with giving an account of such results as recent methods have been successful in establishing.

1. *Methods.*

The methods employed are essentially those developed in connection with simpler hæmolytic systems, and especially in connection with amboceptor-complement systems (Ponder, 1932, *a*).

(*a*) Cell suspensions. Most of the experiments to be described were carried out with systems containing ox cells, for less agglutination occurs with the cells of the ox than with sheep or human cells. The cell suspension is prepared from the thrice washed cells of 4 c.c. of ox blood, finally suspended in 80 c.c. of 0.85 per cent. NaCl. It must be freshly prepared.

(b) Complement. Guinea-pig complement was used throughout, and the same precautions were taken to prevent "decay" as in previous work with amboceptor-complement systems.

(c) Colloidal silicic acid. Not all preparations of this sensitizing agent are satisfactory. I have obtained the best results by using a preparation of ethyl silicate made by Schuchard* ; 16 gm. of this substance are mixed with 400 c.c. of distilled water and boiled gently under a reflux condenser for 2 hours, at the end of which time the mixture is filtered, evaporated to a volume of 300 c.c., and bottled in sterile bottles which are kept in the refrigerator. Just before an experiment is to be performed, the preparation of colloidal silicic acid is diluted with isotonic saline so that a series of dilutions 1 in 10, 1 in 20, . . . 1 in 100, 1 in 120, . . . , 1 in 300, 1 in 400, . . . , 1 in 1000 are available.

(d) The hæmolytic systems. These are prepared so as to have a total volume of 2 c.c., and (in a typical experiment) are made up in the following way. To 0.8 c.c. of various dilutions of the sensitizing agent is added 0.4 c.c. of the cell suspension : the mixtures are then allowed to stand in the water bath at 37° for 10 minutes. At the end of this time 0.8 c.c. of complement, in various dilutions, is added, and the time for complete hæmolysis observed. The systems are typically catastoichic (*i.e.*, the result depends on the order in which the components are mixed), and by far the most active systems are obtained by mixing the three components in this order.

The nomenclature used in this paper is the same as that employed in my investigations of the kinetics of amboceptor-complement systems, *e.g.*, $A_{50}C_{100}$ indicates a system composed of 0.8 c.c. of a 1 in 50 dilution of colloidal silicic acid (denoted, because of its similarity to an amboceptor, by A), 0.4 c.c. of the cell suspension, and 0.8 c.c. of complement diluted 1 in 100, the order of mixing being that in which the symbols are written.

2. Sensitizing Agent Varying, Complement Constant.

Curves showing the relation of the time for complete hæmolysis to the quantity of colloidal silicic acid present in the system, the quantity of complement being kept constant, illustrate excellently one of the principal characteristics of these systems, *viz.*, the occurrence of "hæmolytic zones." A typical

* Supplied by A. H. Baird, of Edinburgh, it is essential that the ethyl silicate used shall be kept in small sealed ampoules (20 c.c. or so), for if exposed to the air it undergoes decomposition, develops an acrid odour, and is useless for the preparation of colloidal silicic acid. Good preparations of the sensitizing agent keep for months in the refrigerator.

experiment, for example, gives the following kind of result (complement dilution 1 in 10).

Table I.

Amboceptor	A_{20}	A_{30}	A_{40}	A_{50}	A_{60}	A_{70}	A_{80}	A_{90}
t (minutes)	—	—	18	7.7	7.5	10	21	—

It will be observed that lysis is restricted to a very limited range of colloidal silicic acid dilutions, lysis in minimal time corresponding to one particular dilution, and that if either too much or too little of the sensitizing agent is present, no lysis occurs at all; indeed, in the higher concentrations of the amboceptor, there is often marked agglutination. This state of affairs is entirely different from that met with in immune body-complement systems, and, as will be seen below, makes any complete analysis of the kinetics of the system almost impossible.

3. Complement Varying, Sensitizing Agent, Constant.

Here again hæmolytic zones are obtained, as will be seen from Table II (amboceptor 1 in 50).

Table II.

Complement	C_3	C_4	C_5	C_6	C_8	C_{10}	C_{12}	C_{16}	C_{20}
t (minutes)	—	21	10.4	9.1	9.7	10.7	12.4	16.6	—

The graph corresponding to this table is shown in fig. 1, and, if regarded as a time-dilution curve for complement acting as a lysin in the presence of a

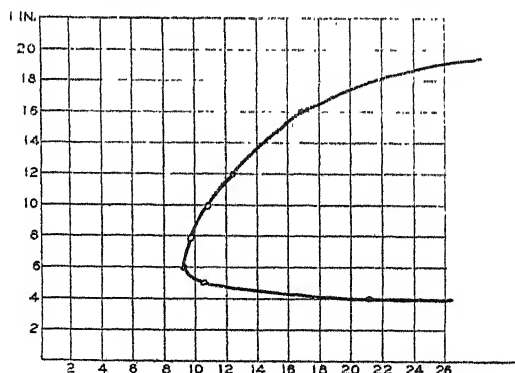


FIG. 1.—Time-dilution curve for complement in the presence of constant quantity of colloidal silicic acid. Ordinate, dilution of complement; abscissa, time in minutes.

constant amount of sensitizing agent, presents two asymptotes, an "upper" and a "lower," between which there is a minimum at which lysis occurs in the shortest time. The form of this curve will be dealt with later.

4. *Sensitizing Agent and Complement both Varying.*

The complicated way in which the quantity of colloidal silicic acid, the quantity of complement, and the time for complete hæmolysis are related is shown in Table III, which gives values of t for a series of colloidal silicic acid and complement dilutions at 37°. The experimental procedure consists in setting up a series of tubes containing 0.8 c.c. of the sensitizing agent in from 1 in 10 dilution to 1 in 160 dilution, adding the cells, and after 10 minutes, 0.8 c.c. of complement 1 in 2 (say); the times for complete lysis are observed. A new series is then set up exactly as before, but now 0.8 c.c. of 1 in 3 complement is added to each tube, and the times again observed, and so on until the table is completed.

Table III.

Silicic acid 1 in	Complement 1 in								
	2.	3.	4.	5.	6.	8.	10.	20.	30.
10	50	—	—	—	—	—	—	—	—
20	16	35	150	75	110	—	—	—	—
30	30	12	16	35	50	130	—	—	—
40	—	50	13	15	28	50	95	—	—
50	—	120	12	8	10	17	45	—	—
60	—	—	130	11	10	12	17	—	—
70	—	—	—	140	45	10	15	160	—
80	—	—	—	—	160	21	30	120	—
100	—	—	—	—	—	—	—	23	—

This table brings out a number of interesting points.

(a) There is a relatively simple relation between the quantity of complement which must be added to cells sensitized with any particular quantity of colloidal silicic acid in order that lysis may occur in the shortest time, for, in general, as the quantity of colloidal silicic acid used for producing sensitization becomes less, the amount of complement which produces the most rapid lysis also becomes less. Plotting the quantity of sensitizing agent against the quantity of complement which gives hæmolysis in minimum time thus results in the type of relation shown by the line marked "minima" in fig. 2; the two quantities, in fact, are linearly related, and so we can put down an expression

which provides us with the amounts of sensitizing agent and complement necessary to give lysis in minimum time,

$$(A - k) = p \cdot C, \quad (1)$$

where A denotes the quantity of colloidal silicic acid used in the system, C the quantity of complement, and k and p are constants. This expression, however, tells us nothing except the quantity of complement which must be used to give as rapid lysis as is possible to obtain with any particular amount of silicic acid, and does not tell us how rapid that lysis will be.

(b) As has already been pointed out, each "hamolytic zone" possesses two asymptotes, one corresponding to the smallest quantity of complement which gives lysis in a very long time (say, 120 minutes), and one corresponding to the largest quantity of complement which gives lysis in about the same time (*cf.* Section 3). We can therefore plot, as in the curve marked "upper asymptote" in fig. 2, the amount of silicic acid against the smaller of these two quantities

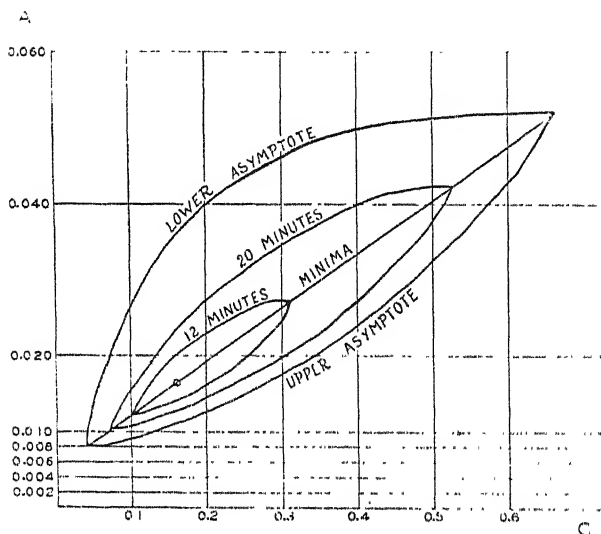


FIG. 2.—The series of ovals referred to in the text. Ordinate, concentration of colloidal silicic acid (arbitrary units); abscissa, quantity of complement (arbitrary units).

of complement, and, as in the curve marked "lower asymptote" in the same figure, the amount of silicic acid against the larger of the two quantities. It is exceedingly difficult to determine these points exactly, for observations which last as long as 120 minutes are apt to be affected by "decay" of complement, and, moreover, the curves often pass to their upper and lower asymptotes very suddenly, so that one may obtain quite rapid lysis with one

dilution of complement and no lysis at all with the dilution next to it; this means that one is liable to miss the exact amount of complement which corresponds to lysis in about 120 minutes unless the steps between the successive complement dilutions are smaller than is practicable.* For these reasons the curve which passes through the points for the two asymptotes is generally rather irregular; we shall see nevertheless that its form is probably that of a peculiar kind of "oval."

(c) Let us now plot against each other the quantities of sensitizing agent and complement which, when used together in these systems, give complete lysis in any specified time, *e.g.*, 10 minutes, 20 minutes, etc. The result is the series of "ovals" shown in fig. 2; these curves are transected, although not symmetrically, by the line for the minima, and lie, like a series of contour lines, within the oval for the asymptotes (*i.e.*, the oval for the specially selected time 120 minutes, supposed to correspond to infinity). As the time for complete lysis is reduced, the ovals become smaller and smaller; they finally shrink, indeed, to one single point situated on the line for the minima, the co-ordinates of this point giving the quantities of sensitizing agent and of complement which give the most rapid lysis possible. In the case of this particular experiment, these quantities are A_{50} and C_5 , which give $t = 8$ minutes.

(d) The graph shown in fig. 2 is thus an asymmetrical three-dimensional figure—the quantities of silicic acid and of complement being two of the co-ordinates and the time for complete lysis the third—and differs in almost every respect from the similarly constructed figure which shows the relation between the same variables in the case of immune-body-complement systems (Ponder, 1932, fig. 4). The treatment of the variables in the conventional way thus yields very perplexing results, and we shall be able to obtain information regarding the kinetics of lysis in these systems only if the experimental results can be simplified (see Section 6), or if we can find some comparatively simple hypothesis which will account for the existence of the ovals (see Section 9).

(e) To express these results in a more familiar form, we can plot the time for complete hæmolysis against various dilutions of complement, the quantity of

* I may mention that experiments of this kind, and, indeed, experiments with colloidal silicic acid in general, are very protracted, for not only is lysis rather slow even at the minima, but the proper concentrations of amboceptor and complement which give any lysis at all are found only after much trial and error. The relations described in this section, for instance, are a kind of composite picture composed by taking together the results of months of work, and only by the rarest good fortune can they all be demonstrated in a single experiment.

sensitizing agent being kept constant for each curve, as in the case of the single curve shown in fig. 1. In this way a series of curves, analogous to time-dilution curves, are obtained, as in fig. 3. It will be observed that each time-dilution curve has two asymptotes, an upper and a lower, with a minimum between them, and, moreover, that the position of this minimum varies with the quantity of colloidal silicic acid used for sensitization: the family thus consists of a series of curves showing "hæmolytic zones," one of these curves having a minimum corresponding to a shorter time than any other, and this corresponding to a particular quantity of sensitizing agent; if the amount of sensitizing agent is greater or less, the corresponding time-dilution curve moves downwards or upwards as the case may be, and at the same time moves to the right, so that the time for the most rapid lysis obtainable becomes longer.

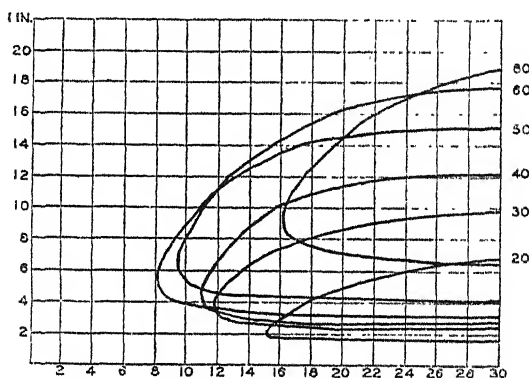


FIG. 3.—Time-dilution curves derived from fig. 2. Ordinate, dilution of complement; abscissa, time in minutes. The figures opposite each curve give the dilution of colloidal silicic acid present in the system.

5. *The Absorption of the Sensitizing Agent.*

It has already been established (Ponder, 1932, *a*) that red cells take up from solution a fraction of the amboceptor present, and that this "absorption" of amboceptor results in their sensitization. It is therefore interesting to establish the fact that the sensitizing agent colloidal silicic acid is similarly absorbed.

The method used is almost identical with that employed for detecting the absorption of amboceptor and is as follows. To 1.6 c.c. of various dilutions of colloidal silicic acid is added 0.8 c.c. of the cell suspension, and the mixture is allowed to stand at 37° for 3 minutes. The tubes are spun on a rapid centrifuge and the cells, which usually show considerable agglutination, thrown down; 0.8 c.c. of the supernatant fluid from each tube is then removed into a series of clean tubes. To each is added 0.4 c.c. of cell suspension; the mixture

is allowed to remain at 37° for 10 minutes, 0.8 c.c. of various dilutions of complement added, and the time for complete lysis observed. The results are then compared with those for a standard system in which 0.8 c.c. of various dilutions of colloidal silicic acid are mixed for 10 minutes at 37° with 0.4 c.c. of cell suspension and 0.8 c.c. of various dilutions of complement added subsequently. In short, two tables similar to Table III are drawn up and compared, the first table being for the quantities of sensitizing agent left unabsorbed in the supernatant fluid from which the sensitized cells are removed, and the second being that for the standard system.

In both tables one finds the usual "zones" and the typical ovals transected by a line for the minima. Two such lines are shown in fig. 4; they make

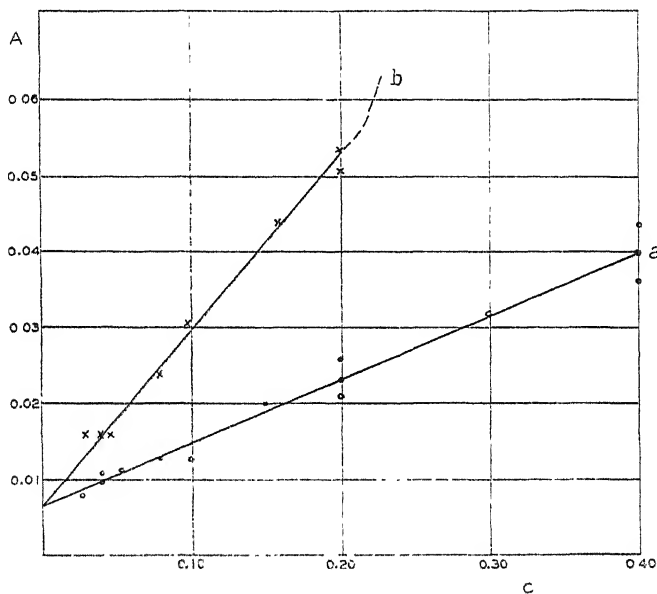


FIG. 4.--Lines passing through the maxima of two systems, to show the absorption of colloidal silicic acid. Curve *a* is that for the standard system; for description see text. The upper part of curve *b* has been dotted because there is some doubt as to its direction. Ordinate, quantity of amboceptor; abscissa, quantity of complement.

the same intercept on the ordinate, but their slopes are different, which indicates that the apparent ratio of sensitizing agent to complement necessary for the production of lysis in minimal time is greater after the sensitizing agent has been allowed to remain in contact with the cells, *i.e.*, that some of the colloidal silicic acid has been absorbed. Within the comparatively small experimental range the amount so absorbed, moreover, appears to be a constant

fraction of the amount introduced, which is a similar result to that already obtained for amboceptor.

While I think that this procedure, and the conclusion to which it leads, is substantially correct, it is disturbing to observe that the family of ovals obtained by plotting the quantities of unabsorbed sensitizing agent against the quantities of complement is different from that obtained in the standard system, particularly in that the "zones" are smaller in the former system and the comparable times for complete lysis usually shorter. The ovals in the former system are thus smaller, and the three-dimensional figure which represents the results in terms of the time variable less flat, than in the standard system; what this means I do not know, but it prevents our comparing the graphical representations of the two systems in their entireties. If we suppose that there is no objection to comparing the two systems with respect to their axes (*i.e.*, with respect to the two lines in fig. 4), this comparison leads to the conclusion already drawn; it should be observed, however, that it is to a certain extent an arbitrary proceeding to compare the graphs of the two systems with respect to their axes alone when they are known to differ in other features, and the result so obtained cannot be supposed to be more than qualitatively correct. We might, for instance, put down the relation

$$(A - k) = \eta (A - k) \quad (2)$$

(as in the case of amboceptor-complement systems, except that k does not appear in these), where A is the amount of sensitizing agent absorbed, A the quantity introduced, and where k and η are constants; but, in view of the differences just mentioned, I doubt if this expression would describe the true state of affairs.

6. *Lysis in Systems Free from Unabsorbed Colloidal Silicic Acid.*

As will be pointed out below, there is evidence that the complex state of affairs shown in figs. 2 and 3 is related to inhibitory effects of the components of the system on each other, and it is therefore important to simplify the systems in every possible way when seeking for a solution for the kinetics of the hæmolysis. One simplification can be made by removing the unabsorbed sensitizing agent, so that the system consists of sensitized cells, complement, and saline, instead of sensitized cells, complement, and saline in which a considerable quantity of unabsorbed sensitizing agent is present.

To a series of tubes is added 0.8 c.c. of the various dilutions of colloidal silicic acid, from about 1 in 10 to about 1 in 600; 0.4 c.c. of cell suspension is

mixed with each dilution, and the mixture allowed to stand for about 3 minutes at 37° in order to allow the sensitization to become complete. The tubes are centrifuged and the supernatant fluid, containing unabsorbed silicic acid, removed; 1.2 c.c. of saline is added, the cells resuspended by gentle shaking, and the tubes again brought to 37°. Complement, in various dilutions, is then added, much as in previous experiments, and the results compared with those obtained in experiments such as described in Sections 2, 3 and 4. Tables IV and V show typical results, in this case after the addition of complement diluted 1 in 10.

Table IV.—Unabsorbed Sensitizing Agent Present.

	A ₁₀	A ₂₀	A ₂₅	A ₃₀	A ₃₅	A ₄₀	A ₅₀	A ₁₀₀	A ₁₄₀	A ₂₀₀	A ₂₄₀	A ₃₀₀	A ₄₀₀
t (minutes)	—	15	8	6	5	11	70	—	—	—	—	—	—

As in the experiments in Sections 2 and 4, there is a distinct “zone” with two asymptotes and a minimum. Compare with this the result shown in Table V.

Table V.—Unabsorbed Sensitizing Agent Removed.

	A ₁₀	A ₂₀	A ₂₅	A ₃₀	A ₃₅	A ₄₀	A ₅₀	A ₁₀₀	A ₁₄₀	A ₂₀₀	A ₂₄₀	A ₃₀₀	A ₄₀₀
t (minutes)	—	—	—	—	—	—	—	—	12	11	12	13	—

In place of the zone in Table IV, we now have a much sharper zone corresponding to much greater dilutions of sensitizing agent, but within this zone the time for complete lysis is virtually independent of the amount of colloidal silicic acid used for sensitization of the cells. Some irregularity in the times is certainly observed in many experiments, but the irregularity is rarely greater than might arise from experimental errors,* and usually substantially the same time for complete lysis is observed over as great a range as from about A₁₂₀ to about A₃₂₀. Outside this range there is usually no lysis at all, although there is often considerable agglutination.

Having found the range within which lysis occurs in this system when C₁₀ is added, let us vary the amount of complement. A remarkable result now appears, for, as the complement dilution is varied, the position of the hæmolytic zone no longer changes; this is in marked contrast to the state of affairs observed in the systems in which unabsorbed colloidal silicic acid is present,

* It is rarely possible, for example, to exclude a certain small degree of hæmolysis on resuspending the cells (see Sections 7 and 8).

in which the position of the zone depends on the complement dilution. The result is well shown in Table VI.

Table VI.—Unabsorbed Sensitizing Agent Removed.

	C_2	C_4	C_6	C_8	C_{10}	C_{12}
A_{160}	—	—	—	—	—	—
A_{200}	5.9	13	21	37	59	—
A_{240}	5.0	12	19	36	52	—
A_{320}	5.5	11	21	34	50	—
A_{400}	5.7	11	23	34	48	—
A_{480}	5.8	12	24	36	60	—
A_{600}	—	—	—	—	—	—

It will be observed that in every case, within the same zone, the times for complete lysis are substantially the same, and that the greater the quantity of complement added, the more rapid is the lysis.* The complement, in fact, seems to be acting like a simple hæmolysin in the range A_{200} to A_{480} , and outside this range there appears to be no lysis at all. We can therefore select one of the rows, *e.g.*, that corresponding to A_{320} , and can plot t against the dilution of complement in the usual manner; this gives fig. 5, which looks like a typical time-dilution curve, and since all the rows are virtually the same, the figures for any one of them give substantially the same curve. Analysing

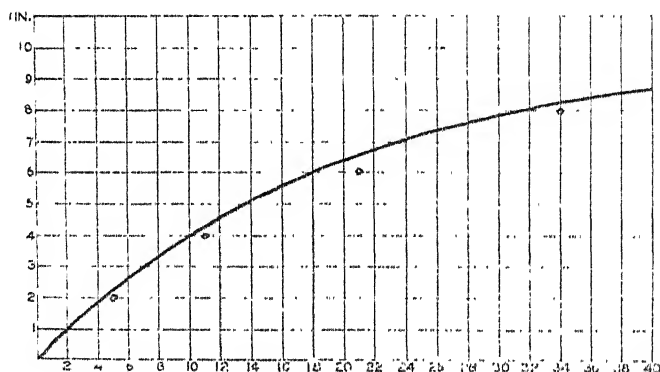


FIG. 5.—Time-dilution curve for complement acting on sensitized cells but in the absence of unabsorbed colloidal silicic acid. Ordinate, dilution of complement; abscissa, time in minutes. The experimental points are marked with circles.

* I am sure that sufficiently careful examination of the rates of lysis within the range would disclose a maximum in the middle of the range and slower lysis corresponding to greater or less, although still effective, amboceptor concentrations. Some evidence of this, indeed, appears in Table VI. But the results are always characterised by some irregularity, and I refer the reader to footnotes on pp. 303 and 307.

this curve we obtain the agreement between experimental and calculated results shown in Table VII by putting $n = 1.1$ and $x = 0.078$ (corresponding to $C_{10.25}$) in the equation

$$kt = \frac{p}{p-1} \left\{ c^{\frac{p-1}{p}} - (c-x)^{\frac{p-1}{p}} \right\}. \quad (3)$$

The complement may thus be supposed to be acting in these simplified systems very much as if it were a simple hæmolysin, and in very much the same way as it has been shown to act in systems containing immune body and complement.

Table VII.

P.	δ .	$t_{\text{exp.}}$	$t_{\text{calc.}}$
12.2	1.250	2.7	2.56
24.4	2.500	6.0	5.44
36.6	3.750	10	8.95
48.8	5.000	14	13.1
61.0	6.250	21	19.8
73.2	7.503	28	26.0
85.4	8.754	37	37.0

It is interesting at this stage to compare fig. 3 and fig. 5. In the former, in which unabsorbed colloidal silicic acid is present in the systems, we have a series of time-dilution curves showing "zones," and a different curve for every different quantity of sensitizing agent. In the latter, in which the unabsorbed silicic acid is removed, we have these atypical curves replaced by a number of more or less typical time-dilution curves, all passing through, or near to, the origin, and all much the same irrespective of the quantity of sensitizing agent used, always provided that this quantity falls within a certain range (in this case, between about A_{200} and A_{430}); if greater or smaller amounts of sensitizing agent are used, there is no lysis at all. This is a much simpler state of affairs than that described in Sections 3 and 4, for, provided that the concentration of the sensitizing agent is suitably adjusted, we have the complement behaving like a simple hæmolysin, and it is not difficult to understand why there should be no lysis if the quantity of sensitizing agent is too small; only one difficulty remains, viz., to explain why there is no hæmolysis when the amount of sensitizing agent is unduly increased. Before doing so, however, I shall deal briefly with two subsidiary matters.

7. *The Effect of p_{H} .*

The preparations of colloidal silicic acid used are quite acid, *e.g.*, colloidal silicic acid diluted 1 in 10 with saline ($p_{\text{H}} = 5.9$) has a p_{H} of 3.7, and, diluted 1 in 100, a p_{H} of 5.8. The recent investigations of Gordon (1933, *a*) have shown the importance of considering p_{H} in relation to haemolytic zones in the case of simple lysins, and I have therefore investigated the possibility of the zones being due to this factor. I have accordingly repeated the experiments of Section 6, the sensitizing agent being prepared at constant p_{H} (6.0, 6.5 and 7.0) in phosphate buffers and in carbonate buffers.

These experiments have been very unsatisfactory, for the resuspension of the cells after removal of the unabsorbed sensitizing agent usually results in lysis when phosphate or carbonate buffers are used for diluting the silicic acid. This lysis occurs quite irregularly, but is sufficient to make the experiments valueless. This, I may remark, is not the only instance of the addition of a sensitizing agent resulting in lysis on the resuspension of the cells; for example, if small (sub-lytic) quantities of sodium taurocholate or glycocholate are added to red cells, the cells thrown down, the supernatant fluid removed, and the cells resuspended in NaCl, lysis promptly occurs.

I have therefore repeated the experiments of Section 4 with the sensitizing agent diluted with buffers, and am satisfied that the appearance of zones is not due essentially to the p_{H} of the colloidal silicic acid.

8. *The Absorption of Complement.*

With amboceptor-complement systems it has been shown that the action of the sensitizing agent is to enable the cells to concentrate complement from the surrounding solution (Eagle and Brewer, 1929; Ponder, 1932, *a*). It would be interesting to establish that such an absorption of complement also occurs when the cells are sensitized with colloidal silicic acid; all my experiments on this point, however, have failed because of the same kind of difficulty as was encountered in the experiments of the previous section.

The method employed for the detection of absorption of complement consists in sensitizing the cells, removing the unabsorbed sensitizing agent, adding known quantities of complement, throwing down the cells at various intervals after this addition, and titrating the complement left in the supernatant fluid. In the case of cells sensitized with amboceptor, this method yields excellent results, and it is possible to show that a considerable fraction of the complement disappears from the suspension medium within a very short time and before any

lysis occurs, and that this quantity is dependent on the amount of the sensitizing agent used. In the case of cells sensitized with colloidal silicic acid, however, some hæmolysis occurs within even a few seconds after the addition of the complement, and it is impossible to throw the cells down sufficiently rapidly; the supernatant fluid is always tinged with hæmoglobin, and in such a fluid it is impossible to titrate complement with any degree of accuracy. Even the addition of isotonic saline, indeed, often results in a small degree of hæmolysis (as in the experiments of the previous section), and, although I have tried in many ways to prevent this, I have been altogether unsuccessful. The sensitizing agent itself, in fact, is too nearly a lysin *per se* to allow of experiments of this kind being carried out.

9. The Hæmolytic Zones.

It will be apparent that the principal difficulty in analysing the curves for colloidal silicic acid-complement systems lies in the appearance of hæmolytic zones, i.e., in the fact that the time-dilution curve for complement is atypical when free sensitizing agent is present. This is the first time that it has been necessary to consider the kinetics of lysis in systems presenting zones, and so I shall deal with the analysis from quite a general point of view.

Consider the case of complement acting in the presence of a constant quantity of sensitizing agent, some of which is free in the system (e.g., curve *b* in fig. 6).



FIG. 6.—Curve *b*, typical time-dilution curve for complement acting in the presence of unabsorbed sensitizing agent; curve *a*, hypothetical curve for complement acting in the absence of unabsorbed sensitizing agent.

If the free sensitizing agent were removed, this time-dilution curve would be of the usual form and would be described by expression (3) with a value of n of about 1.1 (see Section 6). Let us draw such a curve (e.g., curve *a* of the

same figure), assigning, for the moment, any convenient values to x and k , and let us suppose that this is the time-dilution curve for complement acting in the same system but in absence of the free silicic acid. Now, assuming that the free sensitizing agent is responsible for the difference between the curves, let us find a function by means of which we can derive curve b from curve a .

Assume that the free sensitizing agent combines irreversibly with a quantity Δ of the lysin to render the latter inert. By familiar methods (Ponder, 1923, 1925, 1932, *b*, Gordon, 1933, *a*) a relation between $\Delta (= c_1 - c_2)$ and c_1 can be found: call this $\Delta = F(c_1)$. In the two curves under consideration, this is a curve concave to the Δ -axis, as shown in fig. 7 in the curve marked $\mu > 1$. In a sense, of course, this relation has no definite meaning, for x and k in curve a of fig. 6 have been assigned arbitrary values; its general form, nevertheless, enables us to draw some interesting conclusions.

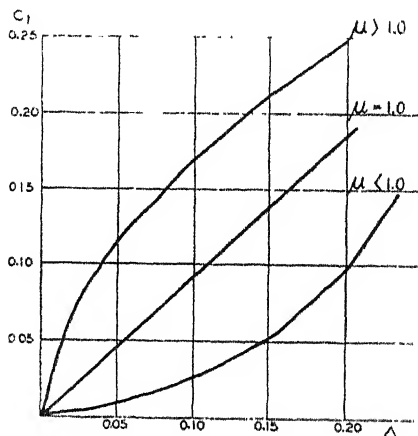


FIG. 7.—The curve $\mu > 1$ is the relation between c_1 and $\Delta (= c_1 - c_2)$ by which curve b of fig. 6 is derived from curve a of the same figure. For further description see text.

1. The first point is that the curve thus obtained (and, indeed, any curve obtained in a similar manner from the time-dilution curves in similar systems) is excellently described by the expression

$$\Delta = \alpha c_1^{1/\mu}, \quad (4)$$

and is therefore similar to the curves for the function $\Delta = F(c_1)$ dealt with by Gordon (1933, *b*) in the case where a quantity of lysin is rendered inert by serum or plasma. Thus we have two types of inhibition both due to the removal of lysin from the system as an inert compound, described by essentially similar formulæ.

2. Consider the forms which expression (4) may take.

(a) If $\mu > 1$, the curve is concave to the Δ -axis, and zones occur. For $\Delta = 0$ when $c_1 = 0$, $t = \infty$ when $(c_1 - \Delta) = c_2$ (which occurs twice owing to Δ increasing with the μ th power of c_1 ; this gives the two asymptotes), and t is at a minimum when $(c_1 - \Delta)$ is at a maximum. The zone therefore results from the particular relation between the quantity of lysin c_1 introduced into the system and the quantity Δ which is rendered inert. This is the system encountered in colloidal silicic acid-complement systems when free sensitizing agent is present.

(b) If $\mu < 1$, the curve is convex to the Δ -axis, and zones cannot occur. The time-dilution curve for the system containing the inhibitor is then uniformly concave to the t -axis; this occurs in the inhibition of a simple lysis by serum or plasma proteins, and I refer the reader to a paper by Gordon (1933, a) in which this form of inhibition is discussed at length.

(c) If $\mu = 1$, $\Delta (= c_1 - c_2) = kc_1$, and c_1/c_2 is a constant. This relation, although occurring frequently in cases where an inhibitor or an accelerator changes the resistance of the system, has yet to be described for a case in which inhibition occurs as a result of irreversible neutralization of lysin.

Although the relation between c_1 and Δ (expression (4)) is unsatisfactory in the sense that it is altogether empirical, it is interesting to find all the known cases of inhibition by the inactivation of a lysin described by the same type of expression, and it is particularly interesting to find those time-dilution curves which present hæmolytic zones susceptible to such a simple type of analysis. The occurrence of such zones is very common in complex hæmolytic systems and also in systems containing precipitins and agglutinins, and I have little doubt that they are usually due to the inactivation of the lysin, agglutinin, or precipitin by an excess of a second component of the system. I may also mention that the zones met with in the case of some of the simple lysins (*e.g.*, sodium glycocholate) can be treated in exactly the same way, and that analysis shows that the zone arises from a quantity of the lysin being rendered inactive, the relation between Δ and c_1 being $\Delta = \alpha c_1^{1/\mu}$, and μ being greater than unity. (See Gordon, 1933, b.)

3. This method of analysis ought, ideally, to be confirmed and developed in at least two respects. (a) It ought to be shown that the subtraction $\Delta = c_1 - c_2$, is valid, as it must be if the lysin is rendered inert by the presence of free silicic acid (see Ponder, 1932, b). (b) The various time-dilution curves showing zones (*e.g.*, those in fig. 3) should all be analysed individually in order to find how α and μ , in expression (4), vary with the amount of sensitizing agent present.

But in order to extend the analysis along either of these lines, we require to know the proper values of α and k to be assigned to the standard curve (e.g., curve α of fig. 6), and these are unobtainable.* A complete analysis of the kinetics of the system is therefore impossible by present methods. These methods, however, have led to certain conclusions of a qualitative kind; these I shall summarize.

(i) When red cells are sensitized with colloidal silicic acid, some of the sensitizing agent is absorbed by the cells, and some is left free (cf. the case of amboceptor, which is identical).

(ii) In systems from which the free sensitizing agent is removed, complement acts as a simple hæmolysin (cf. the identical case of complement acting on red cells sensitized with amboceptor).

(iii) If the free sensitizing agent is present in the system, some of the lysis is rendered inert (cf. the case of any simple lysis which is rendered inert by serum or plasma proteins or by a variety of colloids).

(iv) The relation between the quantity Δ so rendered inert and c_1 , the initial concentration, is $\Delta = \alpha c_1^{1/\mu}$ (cf. the case of the inhibition of saponin or the bile salts by serum).

(v) In the case of colloidal silicic acid-complement systems, $\mu > 1$. Zones therefore occur (cf. the case of sodium glycocholate, although the comparison must be made with caution).

(vi) In general, the greater the quantity of free sensitizing agent, the greater the amount of complement rendered inert (fig. 3) (cf. the inhibition of saponin or the bile salts by serum proteins).

I think, accordingly, that it may fairly be concluded that the present analysis of the kinetics of these systems is adequate, in a qualitative sense at least, and that the complex state of affairs shown in Table III or figs. 2 and 3 is the result of a simple lysis acting on sensitized cells in the presence of a quantity of sensitizing agent which itself reacts with the hæmolysin. It is, of course,

* For purely technical reasons. The steps in order would be (a) to construct a figure such as fig. 4, and so to find η and k ; (b) to construct a figure such as fig. 2 or fig. 3; and (c) to find a time-dilution curve for lysis in the absence of free amboceptor, such as that in fig. 5. Anyone who has worked with these systems will realize that this is an impossibility, in view of the rapidity of decay of complement and of the great duration and considerable uncertainty of the experiments. Even if this were all done, we would be faced with the failure of the experiments in Section 8, for we would never know if the results of the complete analysis were not invalidated by a variation in the extent to which complement is absorbed in the presence of different amounts of sensitizing agent. For these reasons I prefer to leave these problems solved qualitatively rather than to attempt a quantitative analysis which would in all likelihood be erroneous.

just as likely that μ in expression (4) should exceed unity as it is that it should not, and this alone is sufficient to account for the "ovals," the minima, and the hæmolytic zones.

Summary.

This paper is concerned with the kinetics of hæmolysis in colloidal silicic acid-complement systems, in which the silicic acid sensitizes the cells to the lysin complement. This system is characterized by the appearance of "hæmolytic zones," and its investigation by ordinary methods reveals a state of unusual complexity. It is shown, however (a) that the cells "absorb" some of the added sensitizing agent, the remainder being left free in the suspension medium; (b) that in the absence of this unabsorbed sensitizing agent the complement acts as a simple hæmolysin; and (c) that the supposition that the free sensitizing agent reacts with some of the complement accounts for the appearance of the "zones." The relation between the amount of lysin rendered inert as a result of this reaction and the quantity of lysin added to the system, moreover, is of a similar kind to that met with in the case of the inactivation of a simple lysin such as saponin by serum proteins.

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Analytic Studies in Plant Respiration. IV. -The Relation of the Respiration of Potatoes to the Concentration of Sugars and to the Accumulation of a Depressant at Low Temperatures. Part I.—The effect of temperature-history on the respiration/sugar relation.

By JOHN BARKER.*

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Introduction.

The relation of the rate of respiration to the concentration of sugars in the cell is a problem of great theoretical importance. There are some indications in the literature of the effect of high external concentrations of sugar in increasing the respiration, but no full study of the relation of respiration to the internal sugar concentration has yet been published.

For the analysis of the effect of changing internal sugar concentration the potato is a most suitable material, since its excess of hydrolysable starch allows one to alter the sugar content by exposure to cold and so produce a range of sugar between 0·2 per cent. and 7·0 per cent. at will. Moreover it has been recorded that this increase of sugar content is accompanied by increased respiration (Muller-Thurgau, 1882; Hopkins, 1924; Bennett and Bartholomew, 1924).

An extensive study was accordingly undertaken of the relation between the respiration and the sugar concentration, but this relation was soon found to be complicated by a new type of temperature effect, which shows itself as an enduring depression of the respiratory mechanism by exposure to low temperatures. It may be well to state formally that this effect, which we shall speak of as "low temperature depression" and attribute to a special "depressant," is a phenomenon quite apart from that direct retardation of the rate of respiration by low temperature, which is of general occurrence with chemical reactions and lasts no longer than the maintenance of the low temperature.

* This study was begun at the suggestion of Dr. Blackman and carried out under his supervision in the Botany School, Cambridge. A preliminary account was accepted as a thesis for Ph.D. Degree, Cambridge University, 1926. From 1928 the work has been continued at the Low Temperature Research Station, Cambridge.

The special depressant effect may continue for long periods through subsequent high temperatures.*

In the two parts of this communication it is proposed to analyse the interwoven effects of sugar concentration and low temperature depression in determining the rate of respiration, and to establish the conditions which govern the development of the depression. As the respiration/sugar relation cannot be clearly set out without a full understanding of the special characteristics of this type of depression we shall outline our conception of the mechanism in this introduction and assume throughout Part I certain features the evidence for which is postponed to Part II. To avoid confusion in cross-reference the sections, experiments, tables and figures are numbered as one series throughout the two Parts.

In the present investigation the first intention was to determine for a wide range of sugar contents the form of what, for short, we shall always call the R/S curve, *i.e.*, the curve for the relation between the rates of respiration plotted as ordinate axis and concentration of sugar as abscissa axis. The output of carbon dioxide was in general the measure of the respiration, but some special experiments, in which the oxygen intake was also determined, showed that even at the highest respiration rates the respiratory quotient was unity.

With regard to the values for sugar concentration, a general inspection of the accumulated data showed no greater uniformity or regularity for hexoses than for sucrose. On the whole the smoothest results were arrived at by taking the concentration of total sugars, and it is this sum which is always implied by S.

It is not suggested that all three sugars, sucrose, glucose and laevulose, were serving individually as substrate sugars for respiration, but it was decided to treat them as an aggregate for this present stage of the work and later to take up the relation of respiration to the individual component sugars.

The general form of those particular R/S curves which our analysis led us to believe were free from low temperature depression showed a general approximation to the form of an "enzymatic rectangular hyperbola." Were the subject of our study a single enzymatic reaction *in vitro* we might expect a strict conformity to this type, as first expounded by Michaelis. For a complex

* A depressant effect of low temperatures has, so far as we know, never yet been described for any material other than the potato. During the progress of the present work a paper was published briefly recording the effect in the potato (Hopkins, 1924), and though, without critical evidence, relating it to a hypothetical inhibiting effect of the high concentrations of sucrose and hexoses.

process such as respiration *in vivo*, especially when related to a substrate measured by the concentration of a mixture of sugars we must be prepared for a variety of causes of departure of form. When we have surveyed and evaded the chief cause of distortion—the depression just mentioned—we shall be able to evaluate the agreement of the observed R/S form with the ideal (§ 4).

In the analysis of the depression phenomenon the sole measure used is the percentage depression of respiration at a given sugar concentration as compared with the higher normal respiration at the same sugar concentration. The grade of this depression at any moment is determined by the previous temperature experience of the tissues; and once produced the depression seems to be only very slowly reversible within the experimental times and conditions dealt with.

The factors causing this depression are curiously complex, being the outcome of two conditions, which for short we may label with contrasting phrases, the first as *accumulation by cold*, and the second as *development by warmth*, both necessary for the depression effect.

It would be possible to form a chemical or a physical picture of the mechanism of such depression in the living cell. For simplicity we adopt the chemical picture which envisages the steady slow accumulation day after day of an inhibiting substance at low temperatures. With sufficient time this accumulation reaches a limit. The limiting accumulation reached is greater the lower the temperature, very large at -1°C ., moderate at $+1^{\circ}\text{C}$., declining practically to nil in the region of 8°C . This is the first part of the process—the *accumulation by cold*.

For the actual depression of respiration a second condition is needed—that of development—during which the inhibitor, in proportion to its accumulated amount, depresses the respiratory system. This development also takes time, and its rate is strongly affected by temperature; but the temperature relation is the exact opposite of that of accumulation, in that development is very rapid at about 10°C ., quite slow at $+1^{\circ}\text{C}$. and falls to a negligible rate at -1°C .

Clearly then at the lowest temperature, -1°C ., the grade of accumulation day after day can only be actually measured by exposure to a warm temperature for long enough for the complete development of the effect of the accumulated depressant.

At such moderately low temperatures as $+1^{\circ}\text{C}$. and $+3^{\circ}\text{C}$., at which both accumulation and development occur, the individual rates of these two interlocking processes involved in depression can only be made out by careful analysis of the depressant effects of alternating cold and warm temperatures.

In order to guide the reader through the complexity of facts that has been sketched in this introduction we propose to set out the details in a series of separate sections each devoted to one type of relation.

In the course of the investigation, studies with several seasons' crops of potatoes have shown that the R/S forms are essentially similar in different seasons. Accordingly only the data obtained with one stock of potatoes will be given here.

The methods used for determining the respiration* and sugar values† have been described elsewhere (Hanes and Barker, 1931). The carbon dioxide production is stated as milligrams per hour per 100 grams initial fresh weight of potato tissue. The concentrations of both sucrose and hexoses are expressed as invert sugar in percentage of fresh weight.

§ 1. *The Primary Data of Drift of Respiration and of Sugar with Time at various Temperatures.*

In this section it is proposed to set out the data for the drift of respiration and of sugar with time at various temperatures in the form of R/T and S/T curves; in § 2 the R/S forms derived from these data will be considered.

In order to obtain comparable results at the different temperatures a large stock of tubers—King Edward variety—was well-mixed and stored at 10° C. for 2 months after lifting. The samples required for all the experiments given in this paper were then removed from this stock to the low temperatures concerned either on the same date or within a few days of this date.

Throughout the experiments a sample consisted of six potatoes. The values of respiration and sugar content are somewhat variable; on certain occasions measurements were made in duplicate, and both data are recorded in the figures. The curves drawn through the points are usually smoothed.

Experiment 1. Drift of Samples at -1° C., fig. 1.—To obtain data for the drift of respiration and of sugar with time in potatoes changed from 10° C. to -1° C., a large stock of potatoes was transferred to -1° C. and samples were arranged for sugar analysis after periods of 2, 6, 9, 11, 16, 29, 63, 68, 80 and 150 days at -1° C. respectively; in addition the respiration of each sample was determined at the time of analysis. Each sample thus provides a single respiration value—open circles in fig. 1—and a corresponding sugar

* I wish to thank Mr. A. E. Porter for assistance in the experimental work.

† I am indebted to the Hon. Mrs. Onslow for the supervision of the sugar analysis and to Miss N. A. Potter who carried out the estimations.

value—crosses—and from these pairs of individual values two compound curves illustrating the drifts of R and of S with time can be built up.

For the S values a broken line curve is used for all the figures in this section. The corresponding R values, open circles, are not actually joined up into a curve in the figures because they practically coincide with the control respiration curve (black dots with a continuous line) in each figure. These black dots represent the successive R values of a single sample used as a check on the course of R for over 100 days.

From fig. 1 it is evident that up to about 12 days at -1°C . the respiration and the sugar content increase very similarly. After 12 days, however, the

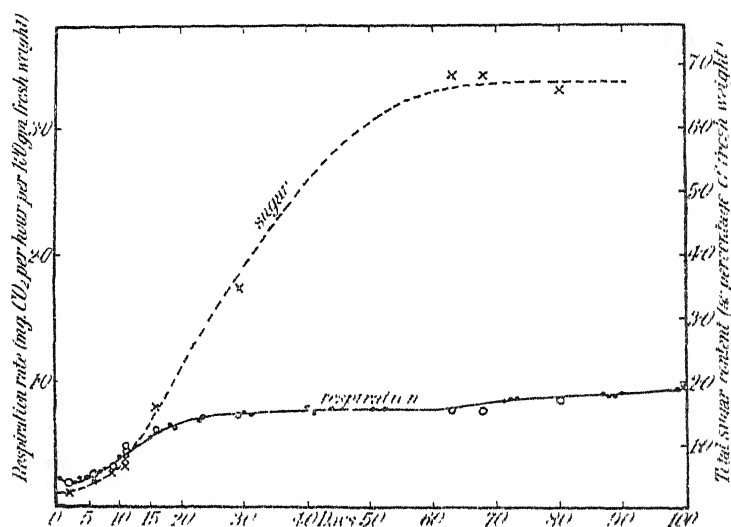


FIG. 1.—Experiment 1. Drifts of respiration and sugar with time in potatoes changed from 10°C . to -1°C .—Samples were taken for determinations of R and S values after 2, 6, 9, 11, 16, 29, 63, 68 and 80 days at -1°C . Values for each sample are shown by circle, R, and cross, S at appropriate time. Respiration of a single control sample measured at intervals is shown by continuous line with black dots.

respiration does not rise as rapidly as the sugar content, and while the respiration is approximately level from 30 to 60 days, the sugar content continues to increase, eventually attaining a maximum amount of about 6.7 per cent. From two further analyses, outside the time range of fig. 1, giving 6.7 per cent. and 7.2 per cent. sugar respectively after 150 days at -1°C ., it appears that the high sugar content attained at about 60 days is subsequently maintained for a long period.

The R/S relation during the first 60 days at -1°C . will be considered in § 2.

The causation of the secondary rise in the respiration occurring after 60–70 days at -1°C . has not been investigated. It must be caused by some factor other than sugar, since the sugar content remains unchanged throughout this second rising phase of respiration.

In the subsequent experiments in this section the methods of obtaining data for the drift of R and of S with time, and the presentation of the data in the figures, are essentially similar to those adopted in experiment 1. Re-statement of the experimental procedure is thus unnecessary; and the detailed legend given in fig. 1 will not be repeated.

Experiment 2. Drift of Samples at $+1^{\circ}\text{C}$., fig. 2, A.—At this temperature, the respiration and sugar increase nearly proportionally for the first 8 days, but after about 11 days, R decreases, while S continues to rise. The increase in S continues for some 50–60 days, the maximum value being roughly half that attained at -1°C .; R reaches a low level after about 30 days.

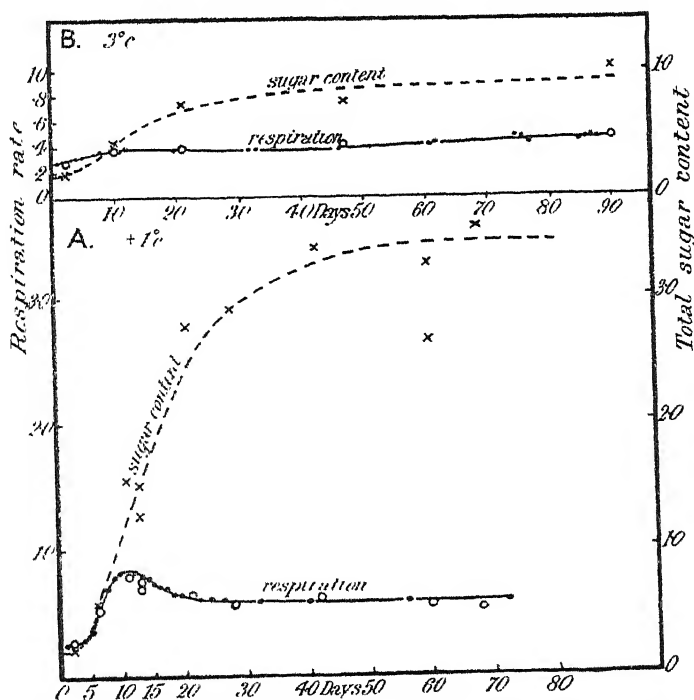


FIG. 2.—Drifts of respiration and sugar with time in potatoes changed from 10°C . A, to $+1^{\circ}\text{C}$., experiment 2. B, to $+3^{\circ}\text{C}$., experiment 3.

The form of the respiration drift is strikingly different from that at -1°C ., and the significance of this difference will be considered in the next section.

Respiration and sugar drifts for $+1^{\circ}\text{C}$. of similar form to those shown in fig. 2, A, have been obtained with potatoes from crops for five different seasons, and also at various times during storage from November to April. These forms are thus a constant characteristic of non-sweet potatoes changed from 10°C . to $+1^{\circ}\text{C}$.

Experiment 3. Drift of Samples at $+3^{\circ}\text{C}$., fig. 2, B.—At $+3^{\circ}\text{C}$. the increase of both R and S is much smaller than at $+1^{\circ}\text{C}$. and -1°C . The drift of R does not show the marked fall observed at $+1^{\circ}\text{C}$.

In these three previous experiments we see that the sugar content gradually increases in potatoes transferred to low temperatures, the maximal concentration attained being higher the lower the temperature. If, however, potatoes rendered sweet by prolonged storage at low temperatures, or partially sweet by storage for short periods, are transferred to 15°C . a rapid desweetening occurs. In the three following experiments we shall investigate the downward drifts of R and of S during desweetening at 15°C . after previous storage at -1°C . or $+1^{\circ}\text{C}$., and to assist the reader to appreciate the time-relations of these three desweetening experiments to the sweetening experiments 1 and 2 already described, the smoothed sugar/time curves for all five experiments are brought together in fig. 3, D.

Experiment 4. Samples at 15°C . after Long Storage at -1°C ., fig. 3, A.—The potatoes had been stored at -1°C . for 57 days and the two sugar values plotted at zero time are the sugar contents of two comparable samples analysed at -1°C . at about this time, and thus show the sugar content at the beginning of the experiment.

On change of temperature to 15°C . the respiration rises rapidly and after 18 hours reaches a peak value of about 5.4, which is roughly seven times the previous respiration at -1°C . Subsequently a marked fall occurs in both R and S, and the data indicate that the fall of R is initially quicker than the decrease of S. After 3 days, however, both R and S show a similar decrease.

Experiment 5. Samples at 15°C . after Short Storage (14 days) at $+1^{\circ}\text{C}$., fig. 3, B.—The peak respiration value at 15°C . and the initial sugar content are much lower than in experiment 4. Subsequently both R and S decrease rapidly at 15°C ., the fall of R being initially more rapid than that of S.

Experiment 6. Samples at 15°C . after Long Storage (56 days) at $+1^{\circ}\text{C}$., fig. 3, C.—Owing to longer storage the sugar content was initially higher than in experiment 5. It is evident that the peak respiration value is roughly the same as in experiment 5, but the rate of fall of R is much slower. Moreover,

there is a marked difference from both the previous experiments, since the fall of R is not initially faster than that of S; in fact R and S appear to decrease at similar rates.

These six experiments provide us with a set of composite curves relating sugar drift to time, and respiration drift to time through sweetening at low temperatures, and desweetening at a high temperature. The curves joining

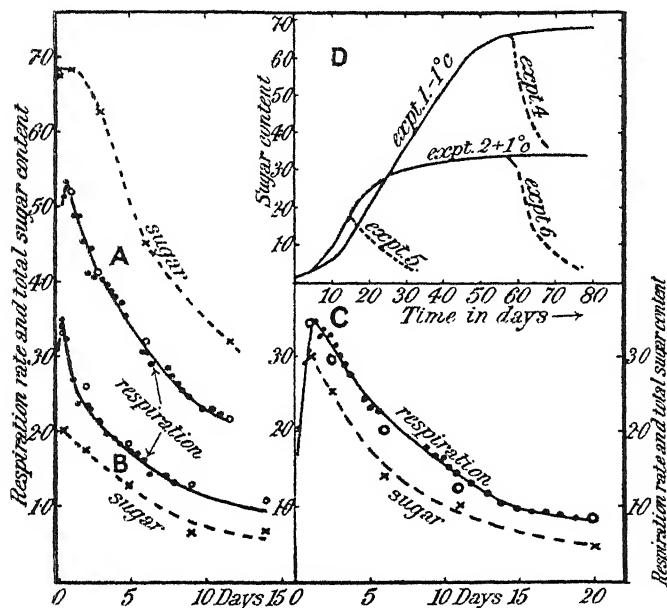


FIG. 3.—Drifts of respiration and sugar with time at 15° C.

A, experiment 4, after long storage at -1° C. B, experiment 5, after short storage at $+1^{\circ}$ C. C, experiment 6, after long storage at $+1^{\circ}$ C. D, time relations of sugar drifts of experiments 1, 2, 4, 5, 6. Experiment 1: sweetening at -1° C. Experiment 2: sweetening at $+1^{\circ}$ C. Experiment 4: desweetening at 15° C. after -1° C. Experiment 5: desweetening at 15° C. after short storage at $+1^{\circ}$ C. Experiment 6: desweetening at 15° C. after long storage at $+1^{\circ}$ C.

up the points show very smooth regular courses, so that we have confidence in proceeding in the next section to study R/S relations, as if we could follow both these features in one piece of tissue simultaneously throughout all the time of its temperature experience.

In the next section we shall also consider the R/S data derived from certain accessory R/T and S/T experiments, *e.g.*, experiments 7, 8, 11, 12. These experiments should properly be included here, but their significance will be

more readily appreciated after the general features of the R/S curves have been surveyed and they are accordingly postponed to § 2.

§ 2. *The Forms Displayed by the R/S Curves Constructed from the Primary Data of § 1.*

The presentation of these experimental results as R/S curves is the most illuminating one for our enquiry into the opposed effects of sugar (S) in raising respiration, and of the inhibitor (I) in depressing it. Before considering the details of the twelve curves, distributed between four temperatures, on which we have now to concentrate, we may draw attention to Table VI, Part II, p. 346, where the derivations of the curves are tabulated, and each R/S curve is assigned a distinctive label so that it may be briefly referred to in discussion without repeated definition. The table is intended as a reference where the reader may ascertain the derivations of any curve should the text not be sufficiently precise.

The twelve R/S curves, each of which is built on a time-series of samples, are recorded together in figs. 4, A and B, and 5, A and B. A general inspection of these curves shows that even for the same temperature the forms and pitches differ markedly; some curves look like rectangular hyperbolas; others have quite different forms.

According to the interpretation put forward in this paper (p. 318), the lowering of the R/S pitch from the highest curve at each temperature, is considered to be due to inhibition of the respiration by a depressant, and the variety of form and pitch shown reflect differences in the grade of inhibition. The curves have therefore been characterized as *highest*, *high*, *intermediate*, or *lowest*, depending on their appearance in the figures. The term *highest* thus implies the maximal or normal R/S relation unaffected by depression; *high* signifies a curve of lower pitch than the *highest*, but in which the grade of depression is only slight or medium; *intermediate* is applied to curves which show an increase of depression in the time-sequence, beginning with only slight depression and terminating with full depression; and *lowest* is used for the curves of minimum pitch in which the depression is fully developed throughout the sugar range.

As a further aid in identifying the curves each is marked with its special temperature, e.g., *highest* at $+1^{\circ}\text{C.}$, or for short *highest* $+1^{\circ}\text{C.}$; for the curves at 15°C. where a previous period of storage at a lower temperature has been given, this temperature is added, viz., *highest* 15° after $+1^{\circ}\text{C.}$, or *highest* $15^{\circ}/+1^{\circ}\text{C.}$

We shall now consider the R/S curves, grouped according to temperature.

Group A. R/S Curves at -1° C., fig. 4, A.

Highest R/S at -1° C. Since a prehistory at a lower temperature than -1° C. cannot be used owing to risk of killing, there is only one R/S curve for this temperature, derived from the R/T and S/T data of fig. 1.

Its form is broadly that of an enzymatic rectangular hyperbola, and shows no indication of the disturbing influence of depression. Moreover, as we shall see later, no evidence that this R/S relation is affected by the depressant factor has been obtained, and we can accordingly accept it as the *highest* and probably normal R/S relation. It is therefore labelled *highest R/S at -1° C.*, and will help as a standard in considering the R/S relations observed at other temperatures.

The degree of conformity with a rectangular hyperbola is considered in Part II, p. 350.

Group B. R/S Curves at $+1^{\circ}$ C., fig. 4, B.

In this group we have three curves which differ markedly in form and pitch and are easily distinguishable by eye as *highest*, *intermediate*, and *lowest*. Their differences are due entirely to previous and current temperature history, so that the details of these experiences must be set out precisely for the three types of form.

Intermediate R/S Curve at $+1^{\circ}$ C., fig. 4, B, based on Experiment 2.—We may first contrast this curve with the form of the curve just recorded for -1° C., and for this comparison the $+1^{\circ}$ C. curve has also been plotted in fig. 4, A, alongside the -1° C. curve.

Both of these curves have had the same type of temperature history. After the preliminary storage at 10° C. common to the whole stock of potatoes, the samples have been kept at either -1° C. or $+1^{\circ}$ C. for months. Though only 2° C. separates these temperatures, yet the forms of respiratory behaviour are quite different. In fact this observation was the original arresting difficulty that faced the investigator and led to the development of a theory of depressant factors as sketched in the Introduction.

Up to about 1.6 per cent. sugar the two curves increase together, the respiration at $+1^{\circ}$ C. for a given sugar content being higher than that at -1° C., as would be expected in view of the higher temperature. Above 1.6 per cent. sugar, however, the $+1^{\circ}$ C. curve falls, and at 3.0 per cent. sugar the respiration is considerably lower than the respiration for this sugar content at -1° C.

An R/S curve of this form was also observed by Hopkins (1924) and attributed to the inhibiting effect of high sugar concentration. This suggestion seems, however, unlikely, since to mention only one reason, no such decrease of R/S pitch occurs at -1°C ., where the sugar is even higher.

We shall show that the fall in the R/S relation at $+1^{\circ}\text{C}$. is due not to the sugar content, but to the low temperature depressant already considered. According to this view the gradual fall of the respiration with time shown in fig. 2, A—from the peak after 11 days at $+1^{\circ}\text{C}$.—is owing to the slowly developing effect of depression. Yet we have to accept that such an effect is quite absent at -1°C .

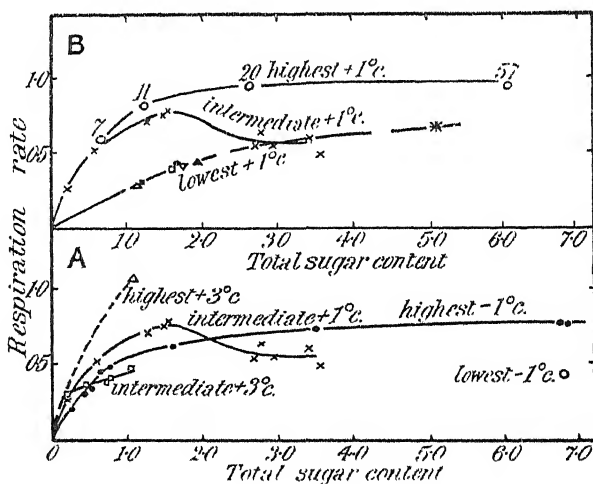


FIG. 4, A.—R/S relations at -1°C ., $+1^{\circ}\text{C}$., and $+3^{\circ}\text{C}$.

- Highest -1°C ., experiment 1. × Intermediate $+1^{\circ}\text{C}$., experiment 2.
- Intermediate $+3^{\circ}\text{C}$., experiment 3. △ Highest $+3^{\circ}\text{C}$., experiment 9.
- Lowest -1°C ., p. 349.

FIG. 4, B.—R/S relations at $+1^{\circ}\text{C}$.

- Highest $+1^{\circ}\text{C}$., experiment 7. × Intermediate $+1^{\circ}\text{C}$., experiment 2.
- ▽, ■, □, ⊙, ▲, △, * Lowest $+1^{\circ}\text{C}$. Tables II, III.

See Table VI, p. 346, for details of the curves.

We do not propose to give all the evidence for this view in the present section, but in order to facilitate the presentation of the R/S relations, we shall assume certain characteristics of depression here for which the detailed evidence will be given later.

Highest R/S Curve at $+1^{\circ}\text{C}$., fig. 4, B, experiment 7.—This experiment was an accessory one consisting of only four determinations of R/S. Samples

which had been kept at -1°C. for periods of 7, 11, 20 and 57 days respectively, and thus showed an increasing sequence of sugar-contents, were transferred to $+1^{\circ}\text{C.}$, and the R/S values determined after $2\frac{1}{2}$ days at that temperature. The S/T and R/T details are not recorded in the text but they could be recovered from the values of S, R and T presented in the figure as the R/S curve labelled *highest* $+1^{\circ}\text{C.}$; the number above each circle gives the days in storage at -1°C.

We wish now to compare the form of this *highest* curve with that of the *intermediate* one at the same temperature. We have seen that the depressant effect does not develop at -1°C. , and in Part II, § 3 we shall show that the effect develops only slowly on transference from -1°C. to $+1^{\circ}\text{C.}$ The R/S values obtained after only $2\frac{1}{2}$ days at $+1^{\circ}\text{C.}$ in this experiment should thus show little, if any, depression.

The form and pitch of the R/S relation exhibited by this *highest* curve at $+1^{\circ}\text{C.}$ confirms this statement. The curve is of enzymatic form, and in marked contrast to the distorted form of the *intermediate* curve at $+1^{\circ}\text{C.}$ shows no indication of depression; so it may be provisionally accepted as the *highest*, and probably normal, R/S relation at $+1^{\circ}\text{C.}$ The agreement in sugar up to 0.6 per cent. between the *highest* and the *intermediate* R/S curves suggests that up to this time in potatoes transferred from 10°C. to $+1^{\circ}\text{C.}$ (*i.e.*, after only 6 days at $+1^{\circ}\text{C.}$) the depressant has not begun to affect the R/S ratio. From this time, however, the inhibition gradually develops, and the R/S relation accordingly falls further and further below the *highest* R/S curve.

Lowest R/S Curve at $+1^{\circ}\text{C.}$, fig. 4, B, based on Experiments 2, 8 and 13 to 17.—It will be shown in Part II, § 3, that the fullest development of the depressant effect at $+1^{\circ}\text{C.}$ may be obtained either (1) by very long sojourn at $+1^{\circ}\text{C.}$, or (2) by a sufficient but shorter period at $+1^{\circ}\text{C.}$ to allow the maximal accumulation of the depressant cause, followed by a short exposure at 15°C. to develop the depression completely, and then a return to $+1^{\circ}\text{C.}$ in the fully depressed state; by this procedure the natural slowness of development at $+1^{\circ}\text{C.}$, is circumvented.

Experiments of these two types are available. Experiments 2 and 8 provide the first type, and experiments 13, 15, 16, 17, described in Part II, § 3, the second type. Summarized experimental details and the R/S values obtained are given in Tables I, II and III. The R/S points of Table I are based on experiment 2 (see fig. 2, A) and form the high sugar part of the *intermediate* curve—fig. 4, B, crosses at about 3.0 per cent. sugar. These points also fall

on the *lowest* curve in this figure, which is made up from the R/S data of Table II, experiments 13 to 17, and from a single point in high sugar, * at 5·2 per cent. sugar, derived from a special experiment—experiment 8, the details of which are summarized in Table III. This experiment was designed to produce higher sugar at $+1^{\circ}$ C. than the equilibrium amount, and this was achieved by an exposure of 28 days at -1° C. preliminary to a long period at $+1^{\circ}$ C. to ensure full depression.

Table I.—R/S Values obtained by Long Sojourn at $+1^{\circ}$ C., Experiment 2.

Type of point in fig. 4, B.	Days at $+1^{\circ}$ C. before analysis.	Respiration.	Percentage total sugar.
Cross	21	0·63	2·77
Cross	28	0·55	2·90
Cross	42	0·60	3·39
Cross	60	0·53	2·67
Cross	60	0·49	3·55

Table II.—R/S Values at $+1^{\circ}$ C. obtained by Forcing the Development, or Depression by Exposures at 15° C.

Experi- ment.	Type of point in fig. 4, B.	Days at $+1^{\circ}$ C. before exposure.	Days exposure at 15° C.	Further days at $+1^{\circ}$ C. after exposure before analysis.	Respiration.	Percentage total sugar.
13	Inverted triangle	10	1	6	0·40	1·76
15	Black spot.....	10	2	6	0·42	1·67
16	Black triangle	10	1	31	0·43	1·94
	Open triangle	14	5	6	0·27	1·16
17	Black square....	81	7	7	0·30	1·19
	Open square	64	5½	29	0·39	1·61

Table III.—R/S Value obtained by Long Sojourn at $+1^{\circ}$ C. after previous Storage at -1° C., Experiment 8.

Type of point in fig. 4, B.	Previous storage at -1° C.	Days at $+1^{\circ}$ C. before analysis.	Respiration.	Percentage total sugar.
Asterisk	28 days	40	0·67	5·20

The *lowest* curve at $+1^{\circ}\text{C.}$, so obtained, may be accepted as showing the fullest development of depression for $+1^{\circ}\text{C.}$, and it will be noted that the curve is broadly of enzymatic form, and in sugar up to about 2.6 per cent. is at a much lower pitch than the *intermediate* curve at $+1^{\circ}\text{C.}$

The above results show the remarkable effect of the temperature history on the R/S relation. By appropriate arrangement of the previous temperature treatment three R/S curves of markedly different form and pitch have been obtained at $+1^{\circ}\text{C.}$ The curves are—firstly, an enzymatic type of *highest* pitch, probably the normal R/S pitch; secondly an enzymatic type of *lowest* pitch, probably showing maximal depression; and thirdly a distorted *intermediate* form, in which the R/S pitch is high (*i.e.*, normal) in low sugar, but with longer time at $+1^{\circ}\text{C.}$ the depression develops, and the R/S pitch falls, eventually reaching the fully-depressed relation of the *lowest* curve.

It will be evident later that by suitable temperature changes still further R/S relations could be obtained at $+1^{\circ}\text{C.}$

Group C. R/S Curves at 3°C. , fig. 4, A.

For this temperature we have two R/S curves of divergent form owing to previous temperature history, which we may distinguish by the usual labels.

Intermediate R/S Curve at 3°C. , fig. 4, A, based on Experiment 3.—This is constructed from the primary data in § 1, the samples being kept continuously at $+3^{\circ}\text{C.}$ without other temperature experience.

The curve is clearly of a distorted type. In 0.20 per cent. sugar the R/S pitch is higher than that for $+1^{\circ}\text{C.}$ as would be expected for the higher temperature, but with increase of sugar the pitch falls, and in 1.0 per cent. sugar is below that of the *highest* curve at -1°C.

Highest R/S Curve at 3°C. , fig. 4, A, Experiment 9.—The effect of the previous temperature experience on the R/S relation has not been investigated so carefully at 3°C. as at $+1^{\circ}\text{C.}$ An R/S value which is probably non-depressed was, however, obtained by keeping potatoes at $+1^{\circ}\text{C.}$ for 6 days, transferring to 3°C. for 2 days and then determining the R and S values.

This R/S point appears in fig. 4, A, as an open triangle at $R = 1.0$ and $S = 1.08$ per cent. We have presented it as joined up with another point, which is actually the earliest value in the series of open squares, which forms the *intermediate* curve at 3°C. just dealt with. This synthetic curve is taken as representing the *highest* undepressed form and probably the normal relation at 3°C.

Group D. R/S Curves at 15° C. after +1° C., fig. 5, A.

Potatoes stored at 15° C. maintain continuously a very low sugar concentration so that no range of S values can be obtained without previous accumulation at low temperatures. As the R/S relations are strikingly different during sweetening at the two low temperatures, -1° C. and $--1^{\circ}$ C., we shall anticipate different behaviours during the subsequent desweetening at 15° C. Indeed, such a variety of R/S relations can be produced at 15° C. according to previous temperature history, that we propose to separate them into two groups. The present group is made for three experiments based on previous exposure at $+1^{\circ}$ C., while Group E will deal with those exposed to -1° C. We may again refer to Table VI, Part II, p. 346, where the derivations of the R/S curves are set out.

Low R/S Curve at 15° C. after 56 days at $+1^{\circ}$ C., fig. 5, A, based on Experiment 6.—We shall associate an R/S curve which is consistently *low* in pitch with a prehistory which has depressed R as fully as possible. One way of obtaining this state would be to keep the samples at $+1^{\circ}$ C. for a sufficiently long time before transference to 15° C. Inspection of the time curve for $+1^{\circ}$ C., fig. 2, A, indicates that 56 days at $+1^{\circ}$ C. should be ample to secure this. This was the prehistory of experiment 6, fig. 3, C, and the R/S values based on that figure are now set out as the *low* 15°/ $+1^{\circ}$ C. curve in fig. 5, A.

Here we are in the presence of desweetening and falling R, so that the sequence of points starts with the highest S and R values and drifts towards zero. Each point is represented by a cross and marked with the number of days that the sample has been at 15° C. up to the analysis.

The form of this curve is clearly something like part of an enzymatic rectangular hyperbola, and does not suggest any distortion of form by depression. While we have only this curve before us for 15° C., we cannot tell whether its pitch is really low, *i.e.*, fully-depressed, but we do learn that there is no depression developing during its sojourn at 15° C., as this should distort the form. Such distortion we shall find presented by the next curve to be considered.

Intermediate R/S Curve at 15° C. after 14 days at $+1^{\circ}$ C., fig. 5, A.—This curve, which is based on experiment 5, fig. 3, B, appears in fig. 5, A, as a series of black dots. It starts with R/S values remote from the previous curve, but in its drift down towards zero R/S comes to coincide with it. The prehistory of this set of samples is that all were kept for 14 days at $+1^{\circ}$ C. before 15° C. This is reckoned as a short period at $+1^{\circ}$ C. compared with the long period of 56 days, and here depression will only be partly developed when the samples

are removed from $+1^{\circ}\text{C}$. The depression will enter upon rapid development at 15°C . and for a few days each sample removed for analysis will be more and more depressed. This the intermediate curve fully demonstrates; the number of days at 15°C . are entered against each point and we see that after 5 days the black spots lie on the *low* curve of crosses showing that by this time they also are fully depressed.

By comparing the pitch of the two R/S curves just considered with the two special points—open squares—in fig. 5, A, at about 0.13 per cent. sugar, it can be readily seen that the two curves are depressed in low sugar. The two special points, experiment 10, show the R/S values of samples of potatoes which had been transferred directly from the main 10°C . stock to 15°C . and kept at that temperature for about 30 days before analysis; not having been exposed to a low temperature they may accordingly be accepted as showing the R/S relation unaffected by depression, see p. 356.

After this indication that the two R/S curves are considerably depressed, we pass to a third R/S curve at 15°C . planned to give the highest pitch attainable in practice.

High R/S Curve at 15°C . after various Times at $+1^{\circ}\text{C}$., fig. 5, A, Experiment 11.—Samples were transferred to 15°C . after being kept at $+1^{\circ}\text{C}$. for 6, 14, 28 and 56 days respectively. In each sample the respiration reached a peak at 15°C . after 16 to 24 hours (see fig. 3, B, for course of respiration of 14-day sample) and the sample was analysed as soon as possible after the peak respiration point was attained.

The R/S values are plotted in fig. 5, A, open circles, the number above each point showing the period in days at $+1^{\circ}\text{C}$. before transference for the one day period at 15°C .; and these points are joined by a broken line to give the *high* $15^{\circ}/+1^{\circ}\text{C}$. curve. The R/S values at 14 and 56 days are also respectively the first points of the *intermediate* $15^{\circ}/+1^{\circ}\text{C}$. curve and the *low* $15^{\circ}/+1^{\circ}\text{C}$. curve.

From this *high* curve, it is evident that the higher sugar of the 14-day as compared with the 6-day sample has resulted in higher respiration, but that the longer periods at $+1^{\circ}\text{C}$., namely, 28 days and 56 days, have only increased the respiration slightly above the 14-day value.

The R/S pitches of the 6- and 14-day samples are markedly higher than the pitch for corresponding sugar content in the *low* curve. These higher values would be expected since, as we have seen, the depressant effect has probably not begun to develop after only 6 days at $+1^{\circ}\text{C}$.; and has only developed slightly after 14 days.

After 28 days the effect of the depressant is fully developed at $+1^{\circ}\text{C}.$, and the sample transferred to $15^{\circ}\text{C}.$ then has almost the same R/S pitch as the 56-day point in which the depression is also completely developed. The early part of this curve after short periods at $+1^{\circ}\text{C}.$ may thus be considered as hardly depressed at all, but it becomes depressed to an increasing extent the higher the sugar content. The curve is therefore labelled *high* but not *highest*. There is no technique for getting this ideal in practice.

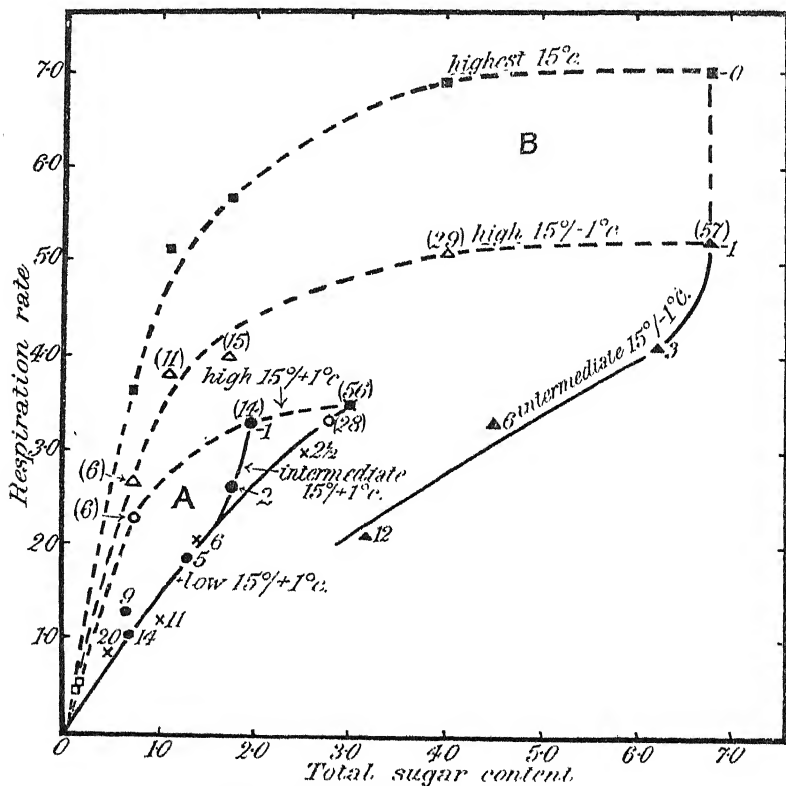


FIG. 5.—R/S relations at $15^{\circ}\text{C}.$

A.

- × Low $15^{\circ}/+1^{\circ}\text{C}.$, experiment 6.
- High $15^{\circ}/+1^{\circ}\text{C}.$, experiment 11.
- Intermediate $15^{\circ}/+1^{\circ}\text{C}.$, experiment 5.
- Undepressed at $15^{\circ}\text{C}.$, experiment 10.

B.

- ▲ Intermediate $15^{\circ}/-1^{\circ}\text{C}.$, experiment 4.
- Highest $15^{\circ}\text{C}.$, p. 334.
- △ High $15^{\circ}/-1^{\circ}\text{C}.$, experiment 12.

See Table VI, p. 346, for details of the curves.

Group E. R/S Curves at $15^{\circ}\text{C}.$ after $-1^{\circ}\text{C}.$, fig. 5, B.

The possible effects of pre-storage at $-1^{\circ}\text{C}.$ have not been so fully explored as those of $+1^{\circ}\text{C}.$, but we can present two types of observed R/S curves,

a *low* one and a *high* one, and finish the set with a calculated *highest* curve.

Intermediate R/S Curve at 15° C. after 57 days at -1° C., fig. 5, B, based on Experiment 4.—The primary data for this curve are in fig. 3, A, while the derived R/S values are represented by black triangles in fig. 5, B, the days that the sample was held at 15° C. before analysis being attached.

During the first 2 to 3 days at 15° C., the R/S pitch falls steeply, but after this period the relation is approximately linear with falling sugar. The curve is thus essentially similar in form to the *intermediate* curve for 15°/+1° C., fig. 5, A. This similarity suggests that as regards the development of the depressant effect potatoes after long storage at -1° C. are closely comparable with potatoes kept only a short period at +1° C., and markedly different from those kept for a long period at +1° C., see the *low* curve at 15°/+1° C. For this reason the curve is labelled *intermediate* though there is no lower one available.

We have seen that after short storage (14 days) at +1° C., a large accumulation of depressant has occurred, but the effect is only slightly developed; transfer to 15° C., however, results in rapid and complete development in 2 to 3 days, producing an initial steep fall of R/S pitch in the *intermediate* curve for 15°/+1° C.

From the initial fall of pitch in the present curve we may thus conclude provisionally that a large accumulation of depressant has occurred at -1° C., but that the effect on the R/S pitch has not developed. On transference to 15° C. the depressant effect is quickly produced and reaches a maximum after about 3 days.

This evidence thus confirms the deduction from the form of the R/S relation during storage at -1° C.—*highest* curve at -1° C. in fig. 4, A—that the depressant effect does not develop at -1° C.

Moreover, the linear part of the present *intermediate* curve from 6.5 per cent. sugar to 3.0 per cent. sugar may be accepted, subject to subsequent confirmation, as showing the R/S relation at 15° C. depressed to the fullest degree for -1° C.

High R/S Curve at 15° C. after various Times at -1° C., fig. 5, B, Experiment 12.—Samples were transferred to 15° C. after storage at -1° C. for 6, 11, 15, 29 and 57 days respectively, and each sample was analysed after 18 to 24 hours at 15° C., *i.e.*, shortly after the respiration had reached the peak at 15° C.—see fig. 3, A, for respiration of a sample kept for 57 days at -1° C. The R/S values are plotted in fig. 5, B, as open triangles, and each point is

marked with the number of days of previous storage at -1°C . It will be noted that the 57-day point of this curve is also the 1-day point of the *intermediate* $15^{\circ}/-1^{\circ}\text{C}$. curve.

From our present curve, it is evident that the respiration is higher after longer time at -1°C ., *i.e.*, with greater sugar content—up to about 29 days, but that extension of the period at -1°C . up to 57 days gives little further increase in the respiration. Moreover, the R/S pitch is markedly higher than in the corresponding curve after various times at $+1^{\circ}\text{C}$., *high* $15^{\circ}/+1^{\circ}\text{C}$., the difference being greater the higher the sugar content. This is in accord with the observations at -1°C . and at $+1^{\circ}\text{C}$., which showed that owing to the negligible development of the depressant effect at -1°C ., the R/S pitch is higher at -1°C . than at $+1^{\circ}\text{C}$.

From the high sugar part of the *intermediate* $15^{\circ}/-1^{\circ}\text{C}$. curve we see, however, that the higher pitch of the -1°C . potatoes at 15°C . is only transitory, and that a rapid development of the depressant effect causes a quick fall in the R/S pitch. A similar fall in pitch would also have occurred in the samples of our present curve of lower sugar content than the 57-day sample if these had been left longer than 24 hours at 15°C . before analysis. The pitch of this curve is thus markedly dependent on the time for which the samples were left at 15°C . before analysis.

The occurrence of such a quick fall in R/S pitch between the 1-day and 3-day points in the *intermediate* curve, suggests that an even quicker decrease in pitch may already have occurred during the first day at 15°C . In Part II, § 4, it will be shown that a large decrease actually takes place, and amounts to a fall in R/S pitch of about 26 per cent. during the first 24 hours at 15°C . We must conclude that the pitch for the 1-day sample of this curve is thus reduced by the depressant by 26 per cent., and the respiration, if the depression were not developed, would be 7.03 , *i.e.*, $\frac{7.03 - 5.2}{7.03} = \frac{26}{100}$. This value has been plotted in fig. 5, black square, at 7.03 respiration and 6.8 per cent. sugar with symbol zero days taking it as the theoretical initial of this *intermediate* curve.

Highest R/S Curve at 15°C . after -1°C .—Evidence of this type is not available for the other points of the *high* $15^{\circ}/-1^{\circ}\text{C}$. curve, but from experiments not given here it is certain that depression develops in the first 24 hours at 15°C . even in samples held at -1°C . for only 6 days. The effect probably does not develop so rapidly in such samples as in fully sweet potatoes, but in the absence of definite evidence a similar rate of development to that for the

fully-sweet sample—the 57-day point—has been assumed, and R/S values for the other samples of this *high* curve, calculated for zero days at 15° C., are plotted in fig. 5, B, and connected to give a *highest* curve which may be taken to indicate the normal R/S relation at 15° C., if development of the depressant effect could be avoided.

This collection of R/S curves at 15° C. affords striking evidence of the profound influence of the temperature history. According to the previous temperature treatment, whether at +1° C. or at -1° C., and whether of short, intermediate or long duration, markedly different R/S relations are obtained at 15° C.

As at the low temperatures, the R/S relations range from maximal and believed normal curves through those of intermediate form and pitch to curves showing minimal R/S pitch, *i.e.*, fullest depression. Moreover the forms and pitches shown at 15° C. strongly confirm the conclusions drawn from the results at +1° C. and at -1° C. as to the behaviour of depression at these temperatures.

Summary.

This first part of our analysis of the respiration/sugar phenomena observed in potatoes contains a collection of records of the empirical relations as these present themselves when the higher sugar concentrations have been obtained by previous exposure to low temperatures. These records establish the fact that there is at work, in a certain class of cases, a very large disturbing factor. A sketch of our conception of the nature of this “low temperature depression” effect has been given in the Introduction. At the end of the further analysis Part II, the conclusions arrived at for the whole investigation will be fully set out.

Analytic Studies in Plant Respiration. V. —The Relation of the Respiration of Potatoes to the Concentration of Sugars and to the Accumulation of a Depressant at Low Temperatures. Part II.—The form of the normal respiration/sugar relation and the mechanism of depression.

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(Communicated by F. F. Blackman, F.R.S.—Received August 30, 1932.)

In Part I of this communication we presented a preliminary stage of the analysis of the interwoven effects of sugar concentration and "low temperature depression" in determining the rate of respiration. In this Second Part we have to begin by investigating the conditions that govern the development of depression and then to apply this closer characterisation to the observed facts already set out in Part I. This will involve a good deal of cross-reference and to avoid confusion the sections, experiments, tables and figures of the present Part are numbered as a continuation of the series in Part I.

§ 3. *The Progress and Magnitude of Depression.*

We may recall our view of the nature of the depression process, given in the Introduction, as the bulk of the evidence in support of it will be set out in this section.

We assume that an inhibitor substance accumulates at low temperatures, and that this accumulation proceeds steadily to a limiting equilibrium amount, which amount is greater the lower the temperature of storage.* At low temperatures, however, this inhibitor only develops very slowly its power of depressing the rate of the respiratory process by interaction with the respiratory mechanism, so that the magnitude of the effect lags behind the amount of the cause. This development can, however, be greatly quickened by short periods of exposure to higher temperatures such as 15° C., and much use will be made of this procedure in this section. After this short warm period the samples can be returned to the low temperature for measurement of their depressed rate of respiration. Fortunately, little alteration of sugar content

* The accumulation of soluble sugars in potato tubers provides an example of a mechanism which is affected by temperature in exactly this manner.

occurs with these sudden short changes of temperature, so that the samples may pass through the interpolated 24 or 48 hours at 15° C. without any appreciable change of sugar values.

It is clear then that the observer is not limited to collecting data based on slow drifts of depression at constant temperatures but can expedite the factor of development-rate without disturbing sugar concentration or amount of inhibitor. In this way it is possible to build up evidence of the nature of the mechanism. Where suitable undepressed standards of respiration are available the percentage depression from the standard can be measured through these treatments and plotted as percentage D against time. Such records are the main objective of this section.

The Course of Accumulation and Development of Depression at +1° C.

We may first recall how the appearance of depression of R proceeds when samples are kept continuously at +1° C. (see R/T at +1° C. only, Part I, fig. 2, p. 321). Initially, the respiration rises in accord with the increase of sugar, but opposed to this rise is the accumulation of the inhibitor and the slow development of its effect. After about 12 days this depression process causes a gradual fall of the respiration.

Interpolation of a 24-hour Period at 15° C. in the Course of Storage at +1° C.—We may next show how a short exposure to a high temperature such as 15° C., though it produces no further accumulation of the inhibitor I, may yet rapidly increase the depression of R by quickly developing the full depressant effect of the accumulated inhibitor.

Experiment 13. 24 hours at 15° C. after 10 days at +1° C.—Fig. 6 records the respiration of a sample—A—which was kept at +1° C. for 10 days, transferred to 15° C. for 24 hours, and then returned to +1° C. The respiration of a control sample B, kept at +1° C. throughout, is also given.

The effect of the period at 15° C. on the respiration at +1° C. was most striking. The respiration of sample A fell rapidly in the course of 4 days after the exposure at 15° C. to a value roughly 46 per cent. below the previous respiration at +1° C. This rapid fall contrasts strongly with the slow fall in sample B.

Sugar analysis shows that with this fall of R is associated a small rise of S giving markedly smaller R/S after exposure to 15° C. The extent of the depression of R/S can be seen by reference to the R/S curves set out in Part I, fig. 4, B, p. 326. Here the *intermediate* curve at +1° C. gives 0.78/1.54 as

R/S before, while the *low* curve gives $0.40/1.76$ as R/S pitch 6 days after the period at 15°C .

Experiment 14. 24 hours at 15°C . after 50 days at $+1^{\circ}\text{C}$., fig. 6. —In marked contrast to the previous experiment no appreciable effect on the R/S pitch was observed if the exposure at 15°C . was delayed until after 50 days' storage at $+1^{\circ}\text{C}$., i.e., when the slow fall of R/S pitch, which begins after about 12 days at $+1^{\circ}\text{C}$., was completed. Fig. 6, sample C.

This result suggests strongly that the rapid fall of R/S pitch in experiment 13 is due to the same cause as the slower fall in potatoes kept at $+1^{\circ}\text{C}$. throughout.

Adopting the inhibitor hypothesis of the depression of the R/S pitch, see p. 318, we may interpret these two experiments as showing that after 10 days at $+1^{\circ}\text{C}$. a large accumulation of inhibitor has occurred, but because of the slow rate of the actual inhibition at $+1^{\circ}\text{C}$., its effect, namely, the depression of the R/S relation, is only developed to a small extent. If left at $+1^{\circ}\text{C}$. the inhibition continues at the slow rate appropriate to $+1^{\circ}\text{C}$., and after about 26 days the effect of the inhibitor is completely developed, and no further fall of the respiration occurs either during longer storage at $+1^{\circ}\text{C}$. or exposure at 15°C .

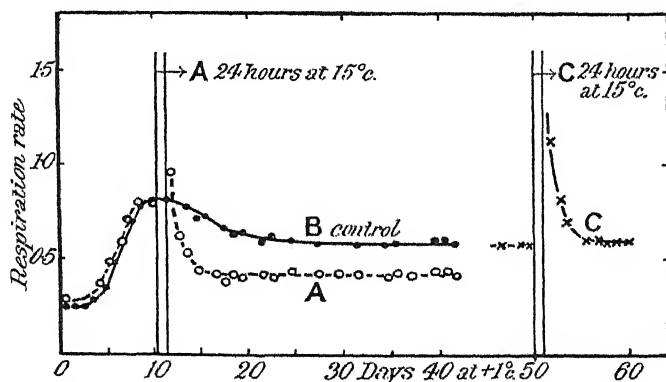


FIG. 6.—Experiments 13 and 14. Effect on respiration at $+1^{\circ}\text{C}$. of exposures at 15°C . for 24 hours.

Sample A, to 15°C . for 24 hours after 10 days at $+1^{\circ}\text{C}$. Sample B, control at $+1^{\circ}\text{C}$. throughout. Sample C, to 15°C . for 24 hours after 50 days at $+1^{\circ}\text{C}$.

On the other hand transference to 15°C . for 24 hours after only 10 days at $+1^{\circ}\text{C}$. allows an extremely rapid development of the inhibition at the higher temperature; and since, as was shown in experiment 15, details of which are given in Part I, Table II, p. 328, a longer exposure at 15°C ., namely, 48 hours,

did not result in an appreciably greater fall of the R/S pitch at $+1^{\circ}\text{C.}$ than 24 hours, the inhibition appears to develop completely during the period of 24 hours at 15°C. ; including necessarily the transitional phase of 4 days at $+1^{\circ}\text{C.}$ before the respiration reaches the depressed $+1^{\circ}\text{C.}$ level.

Moreover, from experiment 16, Table II, it appears that the accumulation of the inhibitor was already maximal 4 days after the treatment at 15°C. , since continued storage at $+1^{\circ}\text{C.}$ for a further 27 days did not result in any further decline in R/S pitch (see Part I, fig. 4, B, *lowest* $+1^{\circ}\text{C.}$, black triangle, $\text{R/S} = 0.43/1.94$, for R/S pitch 31 days after 24 hours exposure at 15°C.).

These deductions are in close accord with the data for the R/S relation at 15°C. given in Part I, see fig. 5, A. Thus in potatoes changed to 15°C. after long storage at $+1^{\circ}\text{C.}$, *low* curve $15^{\circ}/+1^{\circ}\text{C.}$, no sudden fall of R/S pitch occurred in the first few days at 15°C. , since depression had already developed fully at $+1^{\circ}\text{C.}$ On the other hand after only short storage (14 days) at $+1^{\circ}\text{C.}$, *intermediate* curve $15^{\circ}/+1^{\circ}\text{C.}$, a rapid initial fall of R/S pitch occurred, but after 2 days the curve joined the *low* curve, showing that complete accumulation and maximal development of depression had been attained.

From the evidence at $+1^{\circ}\text{C.}$ and at 15°C. we may thus conclude that for $+1^{\circ}\text{C.}$ the maximal accumulation of the inhibitor combined with the complete development of its effect may be obtained either by long storage at $+1^{\circ}\text{C.}$ —(crosses at about 3.0 per cent. sugar in *intermediate* curve $+1^{\circ}\text{C.}$, fig. 4, B) or by a short exposure to 15°C. after a period of 10 days or more at $+1^{\circ}\text{C.}$ —(inverted triangle and black triangle, in the *low* curve at $+1^{\circ}\text{C.}$).

Once the maximal accumulation of inhibitor and the complete development of its effect is produced, the depression appears to be only very slowly reversible in storage at 15°C. Thus the black square at 1.19 per cent. sugar in the *lowest* curve at $+1^{\circ}\text{C.}$ (fig. 4, B, p. 326) shows the R/S pitch at $+1^{\circ}\text{C.}$ after an exposure at 15°C. of 7 days, experiment 17, Table II. The fact that this R/S value falls on the curve—which also passes through the points, namely, the triangles and crosses, shown above to be completely depressed, suggests that the grade of depression has not decreased during the period at 15°C.

As already noted, the form of the *low* R/S at $15^{\circ}/+1^{\circ}\text{C.}$ affords no evidence of a decrease of depression with even longer time at 15°C. , viz., 20 days.* Moreover, in § 5 a method of measuring the accumulation of the depressant will be put forward and it will be shown that as measured by this method the accumulation is approximately constant throughout both the *lowest* curve at

* Analyses after still longer time—34 days, showed an increase of R/S pitch towards the normal, suggesting that a very slow recovery from depression may occur at 15°C.

$+1^{\circ}$ C. and the *low* curve at $15^{\circ}/+1^{\circ}$ C. These curves can accordingly be accepted as showing the maximal grade of depression produced by storage at $+1^{\circ}$ C.

A Quantitative Measure of Depression—Percentage *D* of *R* at same *S*—obtained by Comparison of Undepressed and fully Depressed *R/S* Curves at $+1^{\circ}$ C.—To evaluate the magnitude of the depression which gradually develops during storage at $+1^{\circ}$ C., *intermediate* curve, fig. 4, B, Part I, we may compare the pitch of this curve with the *highest* *R/S* curve at 1° C., which was shown in § 2, Part I, to be probably unaffected by depression. Thus at any given sugar content the percentage decrease of the *R/S* pitch in the *intermediate* curve below the pitch for the same sugar content in the standard *highest* curve is an expression of the percentage depression.

A series of such values, set out against the respective sugar contents is given in Table IV. In addition, since the time-relation of the *intermediate* curve at $+1^{\circ}$ C. is known, we have added to the table values for the duration of storage at $+1^{\circ}$ C., obtained from the smoothed sugar/time curve in fig. 2, A, Part I. This enables us to plot the values both for percentage depression and for sugar content against time—fig. 7—when it becomes evident that the depression is first apparent after about 7 days at $+1^{\circ}$ C., and reaches a maximum after about 26 days.

Table IV.—Percentage Depression of *R* and Increase of *S* during Storage at $+1^{\circ}$ C.

Percentage sugar content	0.5	0.8	1.2	1.6	2.0	2.4	2.8	3.2
Duration of storage in days	5.7	7.7	10.6	13.5	16.8	20.6	26	38
Percentage depression, <i>D</i>	0	4.7	8.2	13.8	24.4	34.0	40	40

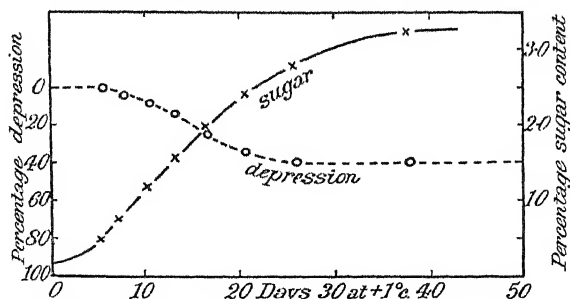


FIG. 7.—Development of depression and increase of sugar content during storage at $+1^{\circ}$ C.

It will be realised that this curve for increase of depression, even apart from the opposing rise of *R* due to increased *S*, is not simply determined either

by the rate of accumulation of the inhibitor or by the rate of development of the inhibiting effect, but by the combined effect of both these rates. As shown on p. 339, the accumulation of the inhibitor is probably nearly maximal after about 10 days at $+1^{\circ}\text{C}$., and the fact that the observed depression at this time is only about 8 per cent., and the full depression is not attained until after 26 days must therefore be due to the slow rate of the development of inhibition.

From this we conclude that the apparent correlation between the development of depression and the increase of sugar shown in fig. 7, is only due to the common slow rate of both these processes at $+1^{\circ}\text{C}$., and cannot be accepted as evidence that the sugar content determines the concentration of inhibitor. The amount of inhibitor is already nearly maximal in 1.2 per cent. sugar.

The progress of the accumulation of inhibitor clearly cannot be determined directly, and could only be analysed by a series of exposures at 15°C . after varying short periods at $+1^{\circ}\text{C}$.

The Course of Accumulation and Development of Depression at -1°C .

Contrasting fig. 1, Part I, giving R/T for -1°C . with fig. 2, Part I, for $+1^{\circ}\text{C}$., we see that there is no sign of a progressive depression at -1°C . We may therefore apply the test of an interpolated period at 15°C . to detect whether there is present any undeveloped potentiality of depression. We have evidence of a positive result in the rapid initial fall while at 15°C . after 57 days at -1°C . in the *intermediate* R/S curve at $15^{\circ}/-1^{\circ}\text{C}$., fig. 5, B, Part I. We now take up experiment 18 in which this matter is fully explored.

Experiment 18. Short Exposures at 15°C . after Long Storage at -1°C ., fig. 8.—A sample -A— which had been kept at -1°C . for 111 days was transferred to 15°C . for 2 days, and returned to -1°C . The change of respiration both at -1°C . and at 15°C . is recorded in fig. 8, and for comparison the record of a control, B, kept at -1°C . throughout, is also given. The period at 15°C . results in a striking fall in the respiration at -1°C . In the course of 5 days after return to -1°C ., the respiration falls to a value 35 per cent. to 40 per cent. below the original -1°C . level, and this low level was then maintained for at least 20 days.

But although the period at 15°C . had such a remarkable effect on the respiration at -1°C ., analysis showed that the sugar content was not appreciably affected by the treatment. The experience at 15°C . thus causes a large fall in the R/S pitch.

Experiment 18 was extended by giving the sample a second exposure of 24 hours at 15° C. 27 days after the first exposure—fig. 8—and a further slight fall in the respiration at -1° C. resulted. Two subsequent exposures again each of 24 hours produced, however, no further fall.

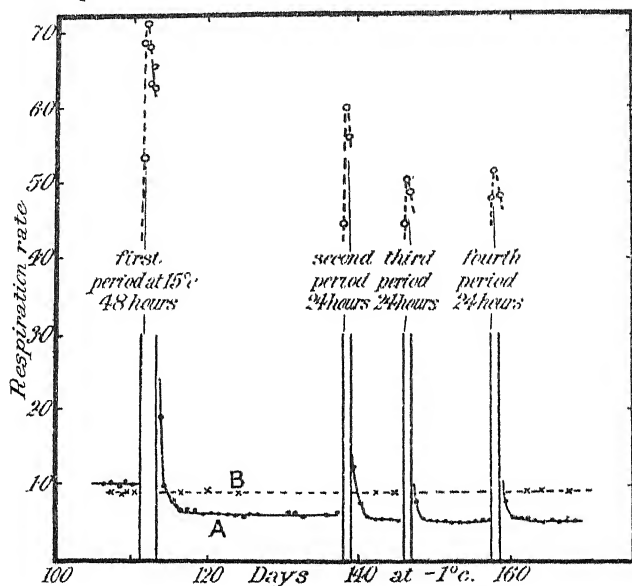


FIG. 8.—Experiment 18. Effect on respiration at -1° C. and 15° C. of successive short exposures at 15° C. after long storage at -1° C. Respiration of Sample A at -1° C. shown by continuous line with solid dots; at 15° C. by interrupted line with open circles. Respiration of control sample B kept at -1° C. throughout shown by interrupted line with crosses.

Since the sugar content is not affected by these short exposures at 15° C. the percentage fall of the respiration from the original level before the first exposure represents the percentage depression of the R/S pitch, and values for depression calculated in this manner from the data in fig. 8 are given in Table V (a).

Table V.—Percentage Depression at -1° C. Produced by Successive Short Interpolated Exposures at 15° C. Experiment 18.

	After 1st exposure.	After 2nd exposure.	After 3rd exposure.	After 4th exposure.
(a)				
Duration of exposure . . .	48 hours	24 hours	24 hours	24 hours
Percentage depression . . .	37	47	49	47
(b)				
Duration of exposure	6 hours	24 hours	24 hours	24 hours
Percentage depression	14.6	28.0	37.5	39.0

A second experiment was made in which shorter periods at 15° C. were used, and the percentage depressions observed are also given in Table V (b).

The data from Table V are recorded in fig. 9 (open circles and crosses) the percentage depression being plotted against the duration of the single period or the sum of the periods for which the sample had been kept at 15° C.

It is evident by the 14.6 per cent. depression after 6 hours at 15° C. that it is the initial hours of exposure which are chiefly effective. The progressive ineffectiveness of succeeding hours involves an exposure of 3-4 days before the maximal depression of 42 to 48 per cent. is reached.

It should be noted that the period required to produce maximal depression would certainly be shorter if it were continuous. Exposure for short periods was adopted in order to avoid the additional complexity of the decrease in sugar, which would have resulted from a continuous exposure of 3 days at 15° C.

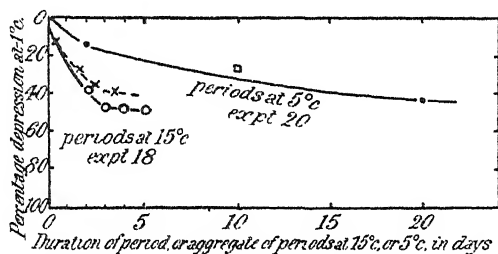


FIG. 9.—Effect of interpolated periods at 15° C. and 5° C. on development of depression at -1° C.

The above results might at first sight seem to show that the fall in R/S pitch was directly due to the exposure at 15° C. It is clear, however, that this is not so, since short exposure at 15° C. has no such effect on the respiration of potatoes stored at 10° C. Moreover, the results are clearly similar to those obtained at +1° C. in experiments 13 and 14, fig. 6.

This state of things suggests that the inhibitor accumulates in storage at -1° C., but its effect is not appreciably developed in the experimental time allowed, namely, 111 days. On transference to 15° C. depression is rapidly completed and we can see its progress on comparing the pitch of the maximum respiration value for successive periods at 15° C. as shown at the top of fig. 8. The respiration pitch falls progressively for the first three periods at 15° C., but between the third and the fourth periods, when the depressant effect is completely developed, no further fall occurs.

As already noted, this rapid initial fall of the R/S pitch on transference from -1° C. to 15° C. is also shown in fig. 5, B, Part I, p. 332, by the *intermediate*

curve $15^{\circ}/-1^{\circ}$ C., and the form of this curve together with the results at -1° C., fig. 8, justifies the conclusion that the complete development of depression is attained after 3 days at 15° C. The question arises whether the maximal accumulation of inhibitor has been attained after 57 days at -1° C., the storage period for the samples of the *intermediate* curve just considered. Unfortunately the full depression at -1° C. produced by an interpolated period of 3 to 4 days at 15° C. was not determined after 57 days at -1° C., but after 74 days at -1° C. an exposure of 24 hours at 15° C.—experiment 19—resulted in a 25 per cent. depression of the respiration at -1° C., a value which is approximately the same as that produced by 24 hours at 15° C. after 111 days at -1° C., experiment 18, fig. 9.

This clearly suggests that the full depression after 74 days would be of the same order as after 111 days, namely, 45 per cent., and indicates that the accumulation of the inhibitor has already reached the equilibrium amount after 74 days.

We may accordingly conclude that the inhibitor is probably also in full equilibrium amount after the slightly shorter period, namely 57 days at -1° C., and that the lower part of the *intermediate* curve at $15^{\circ}/-1^{\circ}$ C. from 6.0 per cent. to 3.0 per cent. sugar, shows the maximum depression for -1° C.

The Rate of Development of Depression at $+5^{\circ}$ C. and the Temperature Coefficient of Inhibition.

The slower rate of development of depression at 5° C. as compared with 15° C. is illustrated by the results (data not set out) of experiment 20 in which samples stored at -1° C. were exposed for periods of 2, 11 and 20 days at 5° C., and then returned to -1° C. to measure the different depressions of R compared with the original value. The values obtained are plotted against the total duration of exposure at 5° C. in fig. 9—dots and open square.

If these points are compared with those for 15° C. open circles and crosses in this figure, it is evident that the rate of development of depression, *i.e.*, the inhibition, has a high temperature coefficient. From these data it is of the order of 6 to 7 for the increase of temperature from 5° C. to 15° C.

Data of this type are not available for changes from $+1^{\circ}$ C. to 5° C., and $+1^{\circ}$ C. to 15° C. It will be recalled, however, that experiment 13, fig. 6, revealed a much more rapid development of inhibition at 15° C. than at $+1^{\circ}$ C., but the data are inadequate for an evaluation of the Q_{10} .

In the single transference from -1° C. to $+1^{\circ}$ C. observed the development of depression was markedly slower than at 5° C., and was negligible in the first 3 to 4 days at $+1^{\circ}$ C.

§ 4. *A Tabulation of the R/S Curves according to Grades of Depression with an Examination of the Form of the Undepressed or Normal R/S Curves.*

In Part I, § 2, we expressed our adoption of the practice of labelling and identifying the set of varying R/S curves obtainable at one given temperature by the terms *highest*, *high*, *intermediate* or *lowest*, according to the pitch of their course in the figures where they are grouped together.

The rationale of this nomenclature is that the lower the curve the greater is the grade of depression. Now that we are entering on a closer analysis of the R/S relations we want to know whether higher and lower curves than those in a given graphic set could have been obtained by appropriate treatment. The specific question we have to ask is whether the *highest* curve presented is really a *maximal curve*, i.e., one in which there is no depression at all; and further is the *lowest* curve presented really *minimal* and therefore as fully depressed as it is possible to get it for the stated temperature. If so, this implies that the maximum equilibrium amount of I has been accumulated and the full development of its depression potentiality has been brought about.

These questions cannot yet be answered quite definitely for every temperature, but views can be adopted for each case and some evaluation made of the depression state of the various intermediate curves. For this survey particulars of all the curves are brought together in Table VI, p. 346, to which reference should be made for the derivations of the curves.

We may first examine the credentials of the high curves to be regarded as *maximal*, and then those of the low curves to be *minimal*. Finally we add an examination of the form of the approved maximal curve and investigate its departure from the type known as the "enzymatic rectangular hyperbola."

Examination of the Highest Curve at each Temperature.

The *highest* R/S curves at -1°C. , $+1^{\circ}\text{C.}$, $+3^{\circ}\text{C.}$ and $+15^{\circ}\text{C.}$, figs. 4 and 5, Part I, pp. 326 and 332, give no indication in their form of any distorting influence of depression. As we have seen, however, the form alone is not a reliable test of whether a curve is really undepressed, and other tests must be applied.

The technique used to get maximum curves is to load up with sugar at -1°C. , where there is accumulation of inhibitor but no development of depression. Following this preliminary treatment the R/S values are determined at the appropriate higher temperature, the period at that temperature being as short as is practicable in order to hold off the development of depression as far as

Table VI.—Titles, Derivatives and Depression State of the R/S Curves.

Temperature.	Title.	Days at temperature.	Pre-history.	Depression state.		Primary data. Expt. Fig.	R/S data, fig.
				Amount of inhibitor.	Development of depression.		
-1° C.	Highest -1° C.	1-68	Only at -1° C.	Increasing to equilibrium	No development	I	4, A
	Lowest point -1° C. . . .	68	Interpolated period of 3 days at 15° C.	Equilibrium amount	Full development	p. 349	4, A
+1° C.	Highest +1° C.	2½	7-57 days at -1° C.	Increasing to equilibrium	No development	7	4, B
	Intermediate +1° C. . . .	1-68	Only at +1° C.	Increasing to equilibrium	Partial to full	2	4, B
	Lowest +1° C.	(1) Long storage at ÷ 1° C. or after -1° C., or (2) with interpolated period at 15° C.		All equilibrium amount	All full	Tables I, II, III	4, B
	Highest +3° C.	2	6 days at ÷ 1° C.	Some inhibitor	No development	9	4, A
+3° C.	Intermediate +3° C.	1-90	Only at ÷ 3° C.	Increasing to equilibrium	Partial to full	3	4, A
+15° C.	High 15°/+1° C.	1	6-56 days at ÷ 1° C.	Increasing to equilibrium	Partial to full	11	5, A
	Intermediate 15°/+1° C.	3-14	All 14 days at ÷ 1° C.	All equilibrium amount	Partial to full	5	5, A
	Low 15°/+1° C.	1-20	All 56 days at -1° C.	All equilibrium amount	All full	6	5, A
					pre-development		
	Highest 15°/-1° C.	Calculated curve for zero depression in high 15°/-1° C.		Allowing for 26 per cent. depression	—	p. 334	5, B
	High 15°/-1° C.	1	6-57 days at -1° C.	Increasing to equilibrium	Partial to 26 per cent.	12	5, B
	Intermediate 15°/-1° C.	1-12	All 57 days at -1° C.	All equilibrium amount	26 per cent. in full	4	5, B

possible. This technique has been used for $+1^{\circ}\text{C}$. and for $+15^{\circ}\text{C}$., but owing to the risk of freezing injury preliminary exposure at a lower temperature cannot be used for -1°C .

In view of this limitation and because the only criterion of the occurrence of depression is the lowering of the R/S pitch from the normal high value, we cannot be certain the *highest* curve for -1°C . is not affected to a slight extent by depression. Nevertheless, in the absence of any contrary evidence and for the purpose of further analysis, we propose to accept it as the normal undepressed R/S relation.

If then this curve is free from depression, the evidence given on p. 344 of the slow development of depression in potatoes changed from -1°C . to $+1^{\circ}\text{C}$., justifies the assumption that the *highest* curve at $+1^{\circ}\text{C}$., obtained with potatoes transferred from -1°C ., is also free from depression.

Owing to an accident with the sample transferred from -1°C . we have to rely for the highest curve at 3°C . on an R/S value obtained by transferring a sample to 3°C . after 6 days storage at $+1^{\circ}\text{C}$. We can, however, be fairly confident that this value is undepressed since it has been shown in § 3 that depression has not developed appreciably after only 6 days at $+1^{\circ}\text{C}$., and little development would be expected in the two days at 3°C . before the R/S value was determined.

As regards the maximal R/S curve at 15°C ., the evidence given in § 3 of the extremely rapid development of depression in potatoes changed from -1°C . to 15°C . shows that the *high* curve at $15^{\circ}/-1^{\circ}\text{C}$., fig. 5, B, is certainly partly depressed. The samples for this curve were transferred from -1°C .; and from fig. 8 it seems probable that considerable depression had developed before the R/S values were determined, namely, after 18–24 hours at 15°C ..

Thus if we consider the respiration during the first period at 15°C . in fig. 8, it is evident that the peak was reached after about 20 hours, and the respiration then decreased roughly 16 per cent. before the return to -1°C . On the other hand the depression shown at -1°C . 5 days after the period at 15°C . was 37 per cent., leaving a difference of 26 per cent. to be accounted for. It can be readily shown that this 26 per cent. depression actually occurs during the first 20 hours at 15°C ., and not during the slow transitional fall of respiration after return to -1°C .

Table VII gives ratios for each up-change from -1°C . to 15°C . in fig. 8 and for each down-change from 15°C . to -1°C . For convenience of reference the increase in percentage depression at -1°C ., caused by each period at

15° C., has been added, and it is evident that while the down-change ratios are all of the same order, averaging 9·7, the up-change ratio for the first period at 15° C., which results in a large development of depression, is considerably below this value. For the third and fourth periods, which produce no appreciable development of depression, the up-change ratios are, however, of the same order as the down-change ratios.

Table VII.—Up-change ratios (maximum respiration at 15° C.)/(previous —1° C. respiration), and down-change ratios (final respiration at 15° C.)/(minimum rate at —1° C.), for each temperature change in fig. 8.

	1st period at 15° C.	2nd period.	3rd period.	4th period.
Up-change —1° C. to 15° C.	7·1	9·4	9·6	9·7
Down-change 15° C. to —1° C.	9·8	10·2	9·6	9·3
Increase in percentage depression at —1° C.	37	10	2	0

This observation, which was confirmed in other experiments of this type, is clearly not in accord with an increase of depression during the transitional phase at —1° C. On the other hand it suggests that the up-change ratio for the first period would also be 9·7 if depression did not develop. With an up-change ratio of 9·7, the peak respiration for the first period at 15° C. would be $9·7 \times 1·0 = 9·7$, and the percentage depression to the observed peak respiration after 20 hours at 15° C. would then be

$$\frac{9·7 - 7·1}{9·7} = 26·8 \text{ per cent.}$$

This figure is in close agreement with the 26 per cent. to be accounted for.

This evidence is the basis of the statement in Part I, § 2, that the 57-day point of the *high* curve 15°/—1° C. is depressed to about 26 per cent.,* and we have used this figure to calculate the non-depressed R/S point shown as a black square at R = 7·03 and S = 6·8 per cent. in fig. 5, B.

The points of the *high* curve 15°/—1° C. in lower sugar are also undoubtedly partly depressed, but since the evidence is not adequate to allow the evaluation of figures for percentage depression in each case, we have calculated the

* It is shown, p. 344, that a 24-hour period at 15° C. would probably produce the same percentage depression after 57 days as after the 111 days of experiment 8.

corresponding non-depressed R/S values assuming a percentage depression of 26 per cent. In this way the *highest* curve at 15° C. has been built up.

From the above it is clear that the *highest* curve at -1° C. is the best-attested maximal and probably normal curve; that if this curve is normal, the *highest* +1° C. and +3° C. curves are also non-depressed; that the calculated *highest* curve at 15° C. is probably non-depressed but that a small percentage error may have been introduced in evaluating the points of this curve in low sugar.

Examination of Lowest Curves at each Temperature.

The technique to obtain minimal curves is to hold the potatoes at the required low temperature for a sufficiently long period to allow the accumulation of inhibitor to reach the equilibrium amount. At +1° C. and +3° C. continued storage will then result in the slow but complete development of the depression potentiality or this development may be rapidly completed by an interpolated period at 15° C. On the other hand depression does not develop during continued storage at -1° C. for the experimental periods investigated here, and an exposure at 15° C. for 3 to 4 days is essential to produce complete development.

No fully depressed R/S points were obtained in the storage period, namely, 68 days, of the final point for the *highest* curve at -1° C. A fully depressed point has, however, been calculated as follows.

In § 3, p. 344, it has been shown that the full equilibrium concentration of inhibitor has probably been attained after 74 days at -1° C., and that an exposure of 3 to 4 days at 15° C. would then produce the complete depression of about 45 per cent. We are justified in assuming that the accumulation of inhibitor would also be maximal after 68 days at -1° C., and that the full depression would again be 45 per cent. The open circle in fig. 4, A, $R/S = 0.42/6.8$, calculated as 45 per cent. depressed from the final R/S point of the *highest* curve can thus be assumed to be fully depressed.

The points of the *lowest* curve at +1° C., fig. 4, B, were all obtained either by prolonged storage at +1° C. or by an interpolated period at 15° C. to hasten the development of complete depression, and in § 3, where the derivation of these points was considered, ample evidence was given that the points all show equilibrium accumulation of inhibitor and maximal development of the depression potentiality. The curve is thus fully depressed at all sugar contents.

At $+3^{\circ}\text{C}$. no attempt was made to obtain fully depressed points in low sugar, but there is evidence that the high sugar part of the *intermediate* curve is minimal. The samples for these points were kept at 3°C . for 21, 47 and 90 days, and assuming rates of accumulation and depression of the same order as at $+1^{\circ}\text{C}$., the maximal depression should certainly have been attained after these storage periods.

Two minimal curves are available at 15°C . The first, *low* $15^{\circ}/-1^{\circ}\text{C}$., was obtained during the desweetening at 15°C . of potatoes previously stored for 56 days at $+1^{\circ}\text{C}$. The equilibrium accumulation of [I] and the complete development of its effect had thus been attained before transference to 15°C ., and as was shown in § 3 there was no evidence of a change of depression during desweetening at 15°C . This curve then shows the maximal depression for $+1^{\circ}\text{C}$.

The second minimal curve is the linear part of the *intermediate* curve at $15^{\circ}/-1^{\circ}\text{C}$. from about 6.0 per cent. to 3.0 per cent. sugar. The potatoes for this curve were kept at -1°C . for 57 days before transference to 15°C ., and the inhibitor was thus probably in full equilibrium amount for -1°C . As shown by experiment 18 depression is completely developed in the first 3 days at 15°C ., and since there is no evidence of a decrease of depression during further storage at 15°C ., the part of the curve from the 3 to the 12-day point may be accepted as fully depressed for -1°C .

From the above we thus see that at each temperature -1°C ., $+1^{\circ}\text{C}$., $+3^{\circ}\text{C}$. and 15°C ., there is good evidence that the *lowest* R/S point or curve obtained shows the equilibrium accumulation of inhibitor and the full development of its effect. In the next section we shall compare these fully depressed curves with the undepressed curves for the same temperature in order to investigate the depression quantitatively.

We do not propose to consider the various *intermediate* curves further. A summary of the depression grade of these curves is given in Table VI.

The Form of the Maximal R/S Curve.

We have seen that of the twelve R/S curves obtained the *highest* R/S curve for -1°C . is best attested as a maximal or normal curve. This curve is accordingly selected for detailed examination to ascertain how closely it conforms with the reaction-rate/substrate concentration relation for an enzyme reaction.

A rectangular hyperbola is now generally accepted as the form of the rate/substrate relation for many enzymatic reactions *in vitro*, and for the present

purpose the equation for the curve may be written :—

$$R = K \times E \times \frac{S}{S + K_m}$$

where R = rate of respiration, S = substrate, *i.e.*, sugar concentration, K = a constant, E = enzyme concentration, and K_m the Michaelis constant.

To obtain the values of $K \cdot E$ and K_m for the rectangular hyperbola of closest fit on the least square method to the highest curve at -1°C ., would be a lengthy process. If the equation is converted into the form

$$\frac{1}{R} = \frac{1}{K \cdot E} + \frac{K_m}{K \cdot E \cdot S}$$

the values of $K \cdot E$ and K_m giving the closest agreement of calculated and observed values of $1/R$ are readily obtained. For our present purpose this second method is sufficient.

The values $K \cdot E = 0.915$ and $K_m = 0.826$ were obtained, and from these were calculated respiration values for each of the observed sugar values. The calculated and the observed respiration values are compared in Table VIII.

Table VIII.—Form of *Highest R/S* Curve at -1°C .

Percentage sugar content.	Observed respiration.	Calculated respiration.	Deviation.
0.23	0.20	0.199	+0.001
0.43	0.30	0.313	−0.013
0.53	0.34	0.358	−0.018
0.64	0.44	0.399	+0.041
0.78	0.47	0.444	+0.026
1.62	0.61	0.606	+0.004
3.51	0.73	0.741	−0.011
6.80	0.76	0.816	−0.056

The departure of observed R from the calculated R values of the closest hyperbola is not random, but has drifts that suggest the observed R values lie on a curve which to some extent departs formally from a rectangular hyperbola, as indeed is only to be expected when the complex metabolism of respiration intervenes between the reactant sugar and the product carbon dioxide.

Another approach that can be made to analyse the relations that control the situation is to adopt the constants of the hyperbola of best fit and calculate for each observed R value the expected value of the respirable substrate S .

These are set out in Table IX. Here we note bigger divergences than in Table VIII, but, of course, at the highest concentrations of the substrate, where the reaction is approaching the limiting rate, no close definition of sugar values can be arrived at.

Table IX.—Comparison of Calculated Values for Respirable Substrate with Observed Total Sugar Values.

Observed respiration.	Observed total sugar.	Calculated respirable substrate.	Deviation.
0.20	0.23	0.231	—0.001
0.30	0.43	0.403	+0.027
0.34	0.53	0.488	+0.042
0.44	0.64	0.748	—0.108
0.47	0.78	0.872	—0.092
0.61	1.62	1.614	+0.008
0.73	3.51	3.26	+0.25
0.76	6.80	4.05	+2.75

The general conclusion that we draw from this brief analytical survey is that the relation of observed R to observed S is some sort of approach to the form of the enzymatic hyperbola over the whole wide range of S from 0.2 to 7.0 per cent., and of R from 0.20 to 0.76 per cent. No such survey of the relation has ever been presented before, and it supports the proposition that enzyme katalysis is the basis of the kinetic control. Any further analysis must be postponed until a more precise view as to the nature and mechanism of production of the actual respirable substrate can be brought forward in place of the empirical value here adopted, which consists of the sum of amounts of the three principal sugars that are detected by chemical analysis.

§ 5. *The Mechanism of Depression.*

Our survey in the previous section of the various R/S curves led us to the view that at each temperature and throughout the full sugar range of each curve, the curve labelled *highest* is probably not depressed at all and that the *lowest* curve is as fully depressed as is possible for the stated temperature; *i.e.*, throughout the *lowest* curve the inhibitor is present in constant equilibrium amount and its effect is completely developed. If then we evaluate the depression of the *lowest* from the *highest*, or normal, curve at each temperature we can obtain values over a fairly wide range of sugar content for the percentage depression produced by a constant amount of inhibitor.

Percentage Sugar Content and Depression.—For this purpose three pairs of curves are available. At $+1^{\circ}\text{C}$., fig. 4, B, Part I, p. 326, we have both a *highest* and a *lowest* curve; at 15°C ., fig. 5, Part I, p. 332, there are two minimal curves, *low* $15^{\circ}/+1^{\circ}\text{C}$. and part of *intermediate* $15^{\circ}/-1^{\circ}\text{C}$., and as a maximal curve with which to compare each of these, there is the *highest* at 15°C . For each pair the *highest* curve has been used as the standard and the percentage depression of the respiration in the *lowest* or minimal curve has been calculated at several sugar points along the smoothed curves. The values obtained are set out in Table X, and in fig. 10 the percentage depression is plotted against the sugar content.

Table X.—Percentage Sugar Content and Percentage Depression.

Percentage sugar content .	1.0	2.0	3.0	4.0	5.0	6.0
Percentage depression $+1^{\circ}\text{C}$	65	51	42.5	37	33	—
„ at $15^{\circ}/+1^{\circ}\text{C}$	68	55	47.5	—	—	—
„ at $15^{\circ}/-1^{\circ}\text{C}$	—	—	66	60	51.5	45

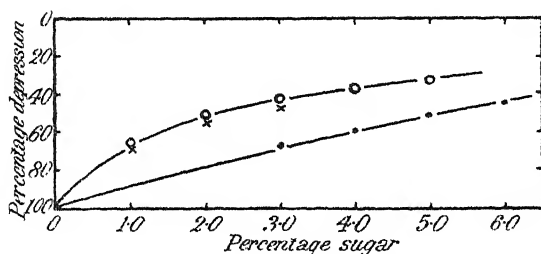


FIG. 10.—Sugar content and percentage depression.

○ at $+1^{\circ}\text{C}$. × at $15^{\circ}/+1^{\circ}\text{C}$. ● at $15^{\circ}/-1^{\circ}\text{C}$.

In considering this figure we may recall that for each set of values the inhibitor is believed to be constant in amount in all sugar contents. If then the inhibitor were non-competitive in type we should expect the percentage depression to be constant with increasing sugar content. This is certainly not so; in fact, the percentage depression falls as the sugar content rises, and the form of the percentage D/S curves suggests, rather, that we are here concerned with a competitive inhibitor.

Measurement of a Depression Coefficient.—A useful measure of the depressant effect is found in the determination of the increase in sugar concentration that is required to nullify the effect of the inhibitor; $S_2 - S_1$ denotes this increase when S_2 is the sugar concentration in the presence of the inhibitor giving the

same rate of respiration as S_1 in the absence of the inhibitor. The ratio $(S_2 - S_1)/S_1$ we may call the depression coefficient— K_D .

Values of K_D calculated for several regions of the three pairs of depressed and non-depressed R/S curves, which were compared in the previous subsection, are set out in Table XI and it is evident that for each pair of curves K_D is fairly constant throughout the given range of sugar content.

Table XI.—Sugar Content and Depression Coefficient, K_D .

	S_1	S_2	$\frac{S_2 - S_1}{S_1} = K_D$
(a) At $+1^\circ$ C. from <i>highest</i> $+1^\circ$ C. and <i>lowest</i> $+1^\circ$ C.	0.2	1.0	4.0
	0.4	1.76	3.4
	0.6	2.7	3.5
	0.8	4.4	4.5
(b) At 15° C. after $+1^\circ$ C. from <i>highest</i> 15° C. and <i>low</i> $15^\circ/+1^\circ$ C.	0.2	0.85	3.25
	0.4	1.49	2.7
	0.6	2.48	3.1
(c) At 15° C. after -1° C. from <i>highest</i> 15° C., and <i>intermediate</i> $15^\circ/-1^\circ$ C.	0.4	3.04	6.6
	0.6	4.52	6.5
	0.8	5.86	6.3

Significance of K_D in the Enzymatic Reaction.—If the relation between rate of respiration and sugar concentration has the simple form $R = \frac{K \cdot E \cdot S}{S + K_m}$ that is set out in § 4, p. 351, and the sugar and inhibitor combine alternatively with the respiratory enzyme, then in the presence of the inhibitor I, the expression for R as a function of S and I is

$$R = \frac{K \cdot E \cdot S}{S + K_m (1 + I/K_i)},$$

where K_i is the dissociation coefficient of the E.I compound. For such a system the depression coefficient K_D calculated as above is equal to I/K_i and independent of S as we have just found from Table XI. To the extent that the respiration relations approach this simple system, just to that extent will K_D be a measure of I/K_i .

The Relation of K_D to Temperature.—Since the dissociation coefficient K_i is not likely to be much affected by small changes of temperature, we may take observed changes of K_D with temperature as a good measure of change of [I], the concentration of the inhibitor at a given temperature when due time has been allowed for the attainment of the maximal equilibrium amounts proper

to the various temperatures. Here again we may say that the proportionality between K_D and $[I]$ becomes closer, the nearer the respiration system approaches a simple enzyme system.

Effect of the Temperature of Storage on the Accumulated amount of the Inhibitor.

--For a measure of the relative amounts of inhibitor at our three temperatures we have then only to arrive at satisfactory evaluations of $K_D = (S_2 - S_1)/S_1$ as previously defined.

The K_D for $+1^\circ \text{C.}$ has been calculated earlier in this section, Table XI, (a), average $K_D = 3.8$ but for -1°C. and $+3^\circ \text{C.}$ we have still to evaluate coefficients.

A fully depressed R/S curve is not available at -1°C. , but a fully depressed R/S point was determined in § 4, and is plotted in fig. 4, A, $R/S = 0.42/6.8$. By comparing this point with the *highest* R/S curve at -1°C. , a K_D for -1°C. has been calculated as follows:—

$$R = 0.42, \frac{S_2 - S_1}{S_1} = \frac{6.8 - 0.64}{0.64} = 9.6.$$

This coefficient is much higher than that at $+1^\circ \text{C.}$ and a similar difference is also shown by the coefficients of potatoes transferred to 15°C. from -1°C. and $+1^\circ \text{C.}$, Tables XI, (b) and (c).

At $+3^\circ \text{C.}$ both a non-depressed curve and an *intermediate* curve of which the high sugar part is fully depressed, are available, fig. 4, A, from which

$$R = 0.4, \frac{S_2 - S_1}{S_1} = \frac{0.76 - 0.3}{0.3} = 1.5.$$

These values of $K_D = [I]$ at -1°C. , $+1^\circ \text{C.}$ and 3°C. are set out in Table XII and plotted against temperature in fig. 11. It is evident that the amount of the inhibitor falls rapidly with increase of temperature.* The extrapolation of the curve clearly suggests that the accumulation of the inhibitor would be small at 5°C. and negligible at 10°C. and 15°C.

Table XII.—Values of K_D at Different Temperatures.

Temperature.	1/T.	$K_D = [I]/K_t$.	$\log K_D$.
-1°C.	0.00365	9.6	0.98
$+1^\circ \text{C.}$	0.00360	3.8	0.58
$+3^\circ \text{C.}$	0.00355	1.5	0.18

* Although the K_D cannot be a true measure of $[I]$ yet it is striking that the relation between the values of K_D and the reciprocals of the absolute temperature $1/T$ is logarithmic (see Table XII).

This is in complete agreement with our experimental observations. In the present series no experiments were made at 5° C., but in other years a small depression was observed in potatoes changed from 10° C. to 5° C.

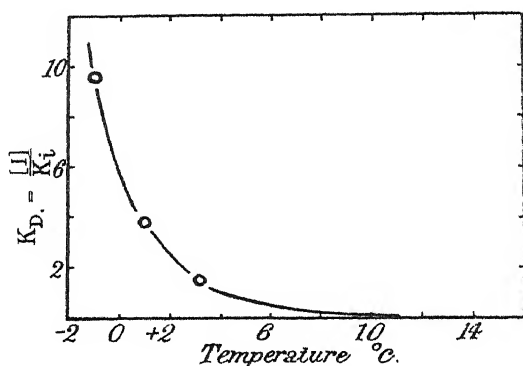


FIG. 11.—Temperature of storage and relative amount of accumulation of depressant.

In potatoes held at 10° C. and 15° C. without exposure to lower temperatures no depression has been observed. Thus the R/S relation remains high in storage at 10° C. or 15° C. for several months, and no fall of R/S pitch is produced at 10° C. by exposure to 15° C. for short periods. Moreover, even after 6 months or more storage at 10° C. the characteristic development of depression occurs on transference to +1° C. suggesting that no depression has arisen at 10° C.

Conclusion.

The main objective of the investigation presented in the two Parts of this communication was the analysis of the form of the relation between respiration and the concentration of extractible sugar, the latter having been varied by exposure of the material at different temperatures. This analysis was found to be complicated by a new type of temperature effect, which consisted in the enduring depression of respiration by exposure to low temperatures. This effect persists for several weeks after return to a higher temperature and so is quite different from the direct retardation of respiration rate by cooling.

For a general outline of this phenomenon, the reader is referred to the introduction to Part I of this paper. There it is stated that the depression of the respiratory mechanism would be attributed to the combined action of two separate processes, both being strongly affected by temperature, but in opposed directions. We have seen how closely the facts conform with this somewhat complicated picture. In § 3 the distinction between the two processes, namely,

the accumulation of the inhibitor and the development of its effect was clearly demonstrated. The grade of the depression was shown to be determined by the previous temperature history, and once produced the depression appeared to be irreversible within the experimental conditions considered. The rate of development of the inhibition was found to have a high temperature coefficient.

In § 2 and 4 the marked influence of the previous and current temperatures on the grade of depression was found to result in a whole series of respiration/sugar curves of widely varying form and pitch. Even so small a difference in temperature as 2° C. in the region near the freezing-point brought about markedly different R/S relations—figs. 4 and 5 and Table VI.

At each temperature the sets of R/S curves ranged from *highest* curves, which were believed to show the normal effect of the sugar content undisturbed by depression, through *intermediate* curves to *lowest* curves, in which the depression was maximal for the particular temperature. These curves demonstrated the remarkable extent to which the respiration could be changed by variation of the sugar content. For example in the *highest* curve at -1° C. the increase in sugar led to an increase to nearly four times the initial -1° C. respiration.

As regards the magnitude of the effect of depression on the respiration, we may recall that with the maximal development at +1° C. the respiration for 2.0 per cent. sugar is decreased by 51 per cent. The depression is thus of considerable magnitude and, moreover, may develop extremely quickly; at 15° C. the respiration may be depressed by as much as 26 per cent. in 24 hours.

In § 4 the form of the *highest* and presumably normal R/S curve for -1° C. was compared with the enzymatic rectangular hyperbola for an enzyme reaction *in vitro*, and found to be closely though not completely conformable with the enzymatic curve. A closer formulation of the significance of the broadly enzymatic R/S relation observed must await a more precise determination of the nature of the actual respirable substrate.

In § 5 the forms of the *highest* or undepressed R/S curves and the *lowest* or fully depressed curves were compared, and the inhibitor was shown to have the characteristics of a competitive inhibitor in an enzyme reaction *in vitro*. Finally a method was put forward of evaluating the *relative* amounts of inhibitor I. The value was 9.6 at -1° C. and decreased to 1.5 at 3° C., suggesting that the accumulation of inhibitor would be small at 5° C. and negligible at 10° C. and 15° C. This evidence shows how markedly the depression is correlated with lowness of temperature.

In conclusion we may note that this paper contains, as far as we know, the first full study that has been published of the respiration/sugar relation in plant tissues.* Not till other studies are available can it be stated whether the approximation to an "enzymatic rectangular hyperbola" is of general occurrence. Nor is there any other recorded example of low temperature depression of respiration available for comparison.

Although the forms of the depressed respiration/sugar curves may yet come to be interpreted otherwise than as due to the activity of a "competitive inhibitor," we have here established the quantitative relations to which any other suggested physical or metabolic interpretation must conform.

I am deeply indebted to Dr. F. F. Blackman for his stimulating interest throughout this study, and for his invaluable assistance in the preparation of this paper. I wish to thank Mr. G. E. Briggs for his advice as regards the enzymatic comparisons.

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* Since this was written there has appeared a detailed investigation of the respiration/sugar relation in the apple by M. Onslow, F. Kidd, and C. West, "Food Investigation Board, Annual Report for 1931," p. 52.

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*The Development of Rana temporaria under the Influence of
Cane Sugar Solution.*

By Madame E. PIPER.

(Abstract by Professor E. W. MacBride, F.R.S.—Received November 16, 1932.)

The main object of the investigations, the results of which are embodied in this paper, was to repeat certain experiments performed by Tornier (1908) and to confirm or confute his conclusions.

Tornier experimented with the eggs of Amphibia—*Rana*, *Bufo* and in particular the Axolotl (*Amblystoma*), keeping them for 48 hours in an 8 per cent. solution of cane sugar, then transferring them for 24 hours to a 2 per cent. solution, and after that to well aerated water in which they completed their development. As a result of this treatment, in further development all sorts of abnormalities, turned up, such as short bodies, swollen abdomens, enlarged mouth cavities, etc. Since similar abnormalities were produced by the use of solutions of glycerine and common salt, Tornier concluded that the energy of the germinal part of the egg had been affected by the reagents used, and that what he termed "plasma-weakness" had been produced, so that the yolky part of the egg, being no longer controlled, swelled up and crushed the more protoplasmic part. He thought that the weakness was immediately due to the cutting off of oxygen by the reagent. He considered that the results which he had obtained with Amphibian eggs explained the production of the "fancy" races of goldfish, because the Chinese breeders, with whom these races originated, were accustomed to rear the spawn of their fish in foul water.

Material and Methods.

The experiments were carried out in the Zoological Laboratory of the Imperial College of Science under the supervision of Professor MacBride.

After preliminary trials, it was resolved to employ a standard solution of 25 per cent. of cane sugar, as this gave decisive results in a much shorter time than weaker solutions.

Pairs of frogs in the sexual embrace were anaesthetised, the eggs extracted and immediately artificially fertilised. After 24 hours of development in clean water the gastrula stage was reached, and then the eggs were immersed in a 25 per cent. solution of sugar.

This lot was divided into four groups, viz. :—

- (a) Which remained in sugar solution for 8 hours and survived 18 days in clean water.
- (b) Which remained in sugar solution for 6 hours and survived 79 days in clean water.
- (c) Which remained in sugar solution for 4 hours and survived 88 days in clean water.
- (d) Which remained in sugar solution for 2 hours and survived 88 days in clean water.

Eggs belonging to the two latter series produced normal larvæ; those belonging to (a) and (b) produced numerous abnormalities. In series (a) the larvæ produced were crippled, warty, and corrugated; 50 per cent. of them became covered with white, and numbers perished prematurely.

The larvæ were fed on pieces of beef and frogs' legs. As a fixative Bouin's fluid gave the best results.

Results.

In general the results of these experiments confirm Tornier's observations; but the explanation which Tornier gave of the *modus operandi* of the reagents is open to serious doubt. Somewhat similar experiments, conducted by Miss Sladden (1932) in the laboratory of the Imperial College, suggest strongly that the injurious effects produced by the solutions are due to their osmotic action in drawing water from the eggs, not to their power of lowering the oxygen content of the water.

Abnormalities closely resembling the "fancy" races of goldfish were obtained.

A few examples of these abnormalities are given below.

(1) *The Lion Head*, resembling the Lion-head variety of goldfish. This variety is so called because the head and opercular region are covered with puffy corrugations of skin which have been fancifully compared to a lion's mane. The movements of the operculum are difficult and respiration is

impeded. The head is shortened, the body swollen and the tail fin doubled. The frog tadpole resembling this variety has dermal sacs covering the whole head down to the gill-cover and a short tail with a wide fin.

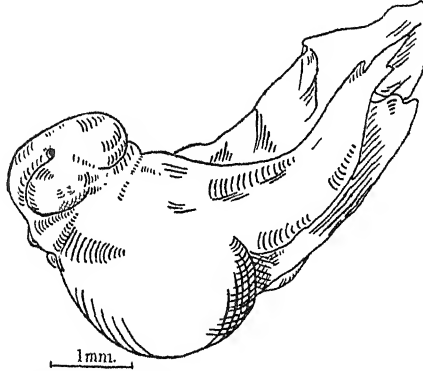


FIG. 1.—A "Lion-head" larva of the 6-hour series, 17 days old.

(2) *The Demi-ranchu or Telescope-fish*.—This variety is so called from its protruding eyes which are like cylinders. The upper lobe of the tail fin is doubled. The body is very short and rounded and the dorsal fin is absent.

The tadpole resembling this is represented in fig. 2. The tail is doubled and there is no fin. Not only the eyes but the nasal and auditory vesicles have taken on the form of bulbous sacs.

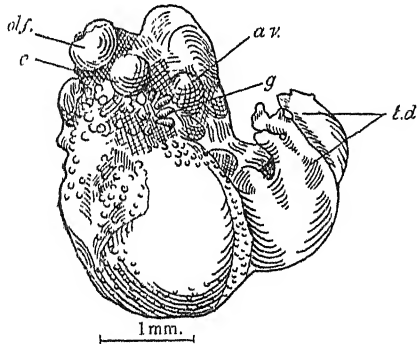


FIG. 2.—"Demi-ranchu" tadpole of 8-hour series, 14 days old. *a.v.*, auditory vesicles *e.*, eye; *g.*, gills; *olf.*, olfactory vesicle; *t.d.*, doubled tail.

(3) *Hydrocephalous Type*.—In this type the abdomen is exceedingly shortened and rounded, and the head is bent upwards at a vertical angle and swollen into a bulbous vesicle. Eyes, olfactory pits, and mouth, are totally absent and the cement organ is shifted to the apex of the head. The tail is absent and the

tail fin represented by a dissected fringe round the hinder border of the abdomen.

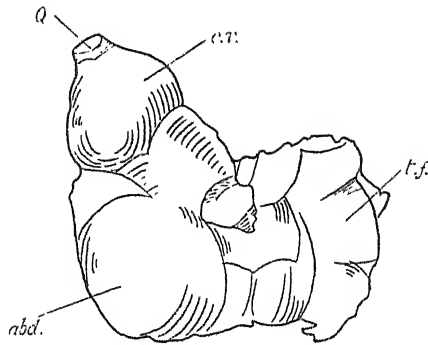


FIG. 3.—Hydrocephalous tadpole of the 8-hour series, 18 days old. *abd.*, abdomen; *c.v.*, cephalic vesicle; *Q.*, cement organ; *t.f.*, tail fin.

(4) *Double-mouthed Form*.—In most respects normal, but two mouths are present and the head is slightly constricted in the sagittal plane.

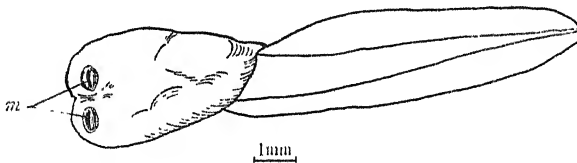


FIG. 4.—Double-mouthed tadpole of the 4-hour series, 23 days old. *m.*, mouths.

If we now consider the tissue modifications produced by the salt solution which lead to the formation of these types we note the following points:—

(a) The ectoderm is much thickened and consists of two or even three layers of cells as compared with the single layer found in normal tadpoles (except in the region of the head). It is frequently thrown into folds. This supports Tornier's contention that the internal organs on which the skin is stretched have failed to keep pace in growth with the normal growth of the skin.

(b) In all types with shortened bodies the notochord is short and stout and its anterior end is swollen and sometimes divided into a double or triple fork. This points to a longitudinal pressure—exactly as Tornier postulated. These abnormal notochords show a central strand of cells containing yolk granules.

(c) Central nervous system.

In the 6- and 8-hour series the olfactory lobes are always, and the cerebral hemispheres often, solid masses. On the other hand the cavities of the fourth ventricle and the spinal cord are enlarged.

(d) Sense organs.

All specimens of the 8-hour series are blind.

The eye may be completely absent or represented only by vestiges, or it may be represented by hollow vesicles, which correspond to the eyes of the telescope goldfish. On the inside of these in one spot a group of pigmented cells represents a rudiment of the retina, and on one spot on the external surface a vestigial invagination represents the lens.

The auditory vesicle, in contrast to the optic and olfactory vesicles, is not much altered. Only in the hydrocephalic larvæ is the vesicle retarded in development; it remains in a primitive condition up to the end of the third week.

(e) Digestive organs.

In 8-hour specimens the mouth opening is not formed at the end of the third week, and the hinder part of the alimentary canal is blocked with yolk.

In 6-hour specimens the front part of the alimentary canal is blocked *but the hinder part is free from yolk*.

One of the most interesting phenomena encountered is the doubling of organs. In "fancy types" of goldfish the tail is doubled and this involves the doubling of the hindermost part of the vertebral column. The anal and caudal fins are also occasionally doubled.

Amongst the abnormal tadpoles doubling is encountered. An individual of superficially normal appearance with a groove in the region of the snout is shown in fig. 4. This is from the 4-hour series.

(f) This specimen proves to have two mouths and four trabecula cranii at the base of the skull.

The notochord is doubled throughout its entire length, and each of the pair carries a double series of myolons. In larvæ of the 6- and 8-hour series a doubled tail is frequently found, and in all these cases the hinder end of the notochord is forked and usually the spinal cord as well. But the hinder end of the notochord is forked in tadpoles with an abnormal tail even though the tail outwardly appears single.

In the specimen with double notochord throughout, the fore-brain (cerebrum), infundibulum and olfactory lobes are all doubled.

Discussion.

The cause of all these irregularities is clearly to be sought in some action of the 25 per cent. sugar solution. Such a solution undoubtedly lowers the oxygen

content of the water but Miss Sladden's work makes it probable that the real cause is the osmotic properties of the solution, causing injury to the delicate cells on the growing blastoporal rim by withdrawing water. These cells according to Spemann (1924) regulate the entire development of the embryo, as is proved by transferring a portion of the blastoporal rim of one newt embryo to the flank of another newt embryo of similar age-- the graft determines the formation of a new spinal cord and notochord. The regulative faculty is weakened and abnormalities result. But the yolk, imbibing water and swelling, as Tornier supposed, comes into play as a secondary factor. We have emphasised the fact that the intestines of abnormal larvæ are full of undigested yolk.

When the larvæ are replaced in clean aerated water, some of the superfluous water absorbed is expelled from the yolk and penetrates to other portions of the body causing huge swellings principally in the lymph sinuses. This causes the huge telescopic eye balls in the "Demi-ranchu" larva; since the cavity of the eyeball is a lymph space, it also gives rise to the huge sub-cutaneous vesicles in the hydrocephalous specimen which literally crush eyes and fore-brain out of existence. According to Hertwig (1894) the hypertonic solution checks the development principally of the yolky cells, which do not differentiate and divide as they should. This is highly probable seeing that yolk is found everywhere—even in the abnormally developed brain vesicles of certain larvæ—where growth has been interfered with.

The doubling of organs was ascribed by Tornier to the doubling of the growing area of young cells owing to the pressure exercised on it by the neighbouring yolk. This on the whole is exceedingly probable. The doubling of a lizard's tail is brought about by this cause and also the doubling of a vertebrate limb.

But it is clear that though osmotic pressure may account for the abnormalities of tadpoles it cannot be the cause of the similar abnormalities of goldfish fry, for although these live in unhealthy water it is not water of increased osmotic pressure. The cause of the similarity must be *injury* to the growing cells owing in the one case to osmotic pressure and in the other case to toxins.

Further, since the abnormalities in goldfish are to some extent hereditary, the injury must affect the young growing cells which give rise to the germ cells. It is now known that mutations can be caused by X-rays and by heat, and there is nothing surprising in the suggestion that they can also be originated by soluble toxins.

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The Biological Action of Homogeneous and Heterogeneous X-rays.

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[PLATE 16.]

The experiments described in this paper were undertaken in order to investigate the following two conclusions drawn by Moppett (1929):—

- (1) That a homogeneous beam of X-rays is enormously more active biologically than is a heterogeneous beam.
- (2) That there is an antagonism between X-rays of different wave-lengths as shown by their biological effects.

Moppett based these conclusions on the effects produced by irradiating the allantois of hens' eggs. He found that the irradiation by X-rays of a portion of the allantois after removal of the overlying shell produced two types of reaction. Moderate doses of irradiation caused hyperplasia and more intense doses atrophy, and he measured the threshold doses needed to produce these effects. He used two types of irradiation, namely, a direct mixed beam of X-rays and a homogeneous beam diffracted from a crystal. Photographic measurements showed the former to be about 1000 times as intense as the latter. He found, however, that in order to produce hyperplasia or atrophy of the allantois, nearly equal durations of exposure with the different types of irradiation were needed.

He therefore concluded that the biological effect produced by a homogeneous beam per unit intensity was over 300 times greater than that produced by a heterogeneous beam. Furthermore he stated that "Direct irradiation contains all the component wave-lengths which might produce atrophy in much greater intensity than the monochromatic rays since the crystal is very inefficient. Considering the great difference between the respective threshold

doses it seems necessary to assume an active neutralisation between the components of mixed radiation in their biological effects."

The fundamental importance of these conclusions was so great that I repeated Moppett's experiments and followed his technique as closely as possible. Moppett's publications subsequent to 1929 (1930, *a*, 1930, *b*, 1930, *c*, 1931, 1932) are based on the conclusions which have been stated, and therefore no further reference to these later papers will be made.

Nature of X-rays Used.

The source of X-rays was a Muller water-cooled hot cathode tube with a tungsten anticathode. This was contained in a lead box in order to prevent interference with the experiment or measuring apparatus by scattered radiations.

Since a kilovoltage of about 70 excites the group of lines of highest frequency in the tungsten K series, a kilovoltage of 78 was used in all experiments in order to ensure the production of the characteristic K radiation of tungsten. The amperage was constant at 5 milliamps and the distance from the anticathode to the target was 77 cm. in all experiments. The apparatus was kept running for 30 minutes immediately before each experiment, after which period the output of X-rays was found to be of constant intensity.

The general method used was to expose an egg, contained in an experimental box, to a narrow beam of X-rays. Three types of irradiation were used, namely, (*a*) direct heterogeneous beam, (*b*) reflected homogeneous beam, (*c*) scattered heterogeneous beam.

(*a*) *Direct Beam*.—The general arrangement of the apparatus is shown in fig. 1. The X-ray beam reached the egg through two narrow slits in lead plates, and the rays were therefore approximately parallel. The mode of treatment of the egg and the arrangement of the experimental box will be described later.

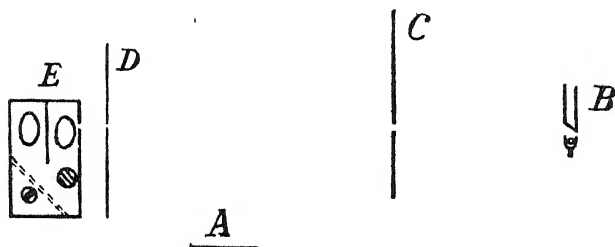


FIG. 1.—Apparatus for direct irradiation.

A, scale = 10 cm.; B, anticathode; C, lead screen with 3 mm. slit; D, lead screen with 2 mm. slit; E, experimental box.

(b) *Reflected Homogeneous Beam.*—In certain experiments homogeneous X-rays were used. These were obtained from a spectrometer which was simple and easy of manipulation and which was a modification of that described by de Broglie and Lindemann (1914). It consisted essentially of a sheet of mica obtained by cleavage and bent into the form of a cylinder. The advantage of this type of spectrometer lay in the curved shape of the mica which allowed a beam of X-rays comprised within a very small angle to strike the crystal in such a way as to produce a large range of possible angles of reflection.

With a slit at S, fig. 2, an extended spectrum was obtained along AB. In order to increase the intensity of the reflected rays even at a sacrifice of resolving power, a large radius of curvature, approximately 70 cm. was used. The reflected beam was received through a slit 2 mm. wide. The exciting beam was

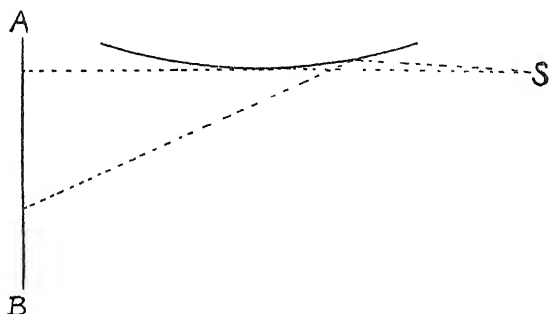


FIG. 2.—Diagram illustrating diffraction from a curved sheet of mica.

produced at a kilovoltage of 78, which ensured the production of the characteristic K radiation of tungsten which has a wave-length of 0.21 Å. Since the grating constant of mica is 10 Å the characteristic K radiation of tungsten is reflected from mica at a glancing angle of approximately half a degree. By experiment the beam reflected at about this angle was found to be more intense than those which were reflected at adjacent angles, and furthermore its mass absorption coefficient in aluminium $\frac{\mu}{\rho_{Al}}$ was found to be 0.24 when the absorption was about 50 per cent. These results suggest that the beam was composed of the reflected tungsten K radiation.

Mr. J. Paton of the Physics Department of this University, kindly tested the homogeneity of the beam. His method was to measure the successive absorptions in very thin sheets of aluminium by means of a tilted electroscope, and he found that the beam was absorbed exponentially in 12 successive sheets of aluminium. While it is likely that reflected radiations of other orders were

present in the beam, the exponential absorption in aluminium suggests that the intensity of these radiations was small compared with the intensity of the tungsten K radiation (Kaye, 1926, p. 104).

The general arrangement of the apparatus in these experiments is shown in fig. 3.

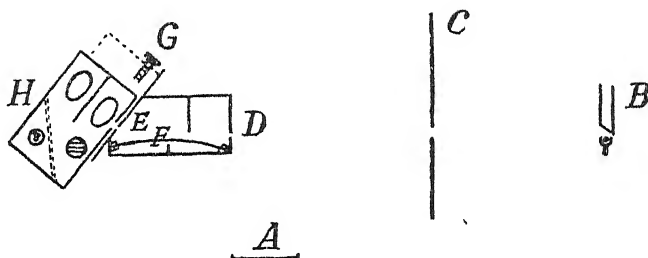


FIG. 3.—Apparatus for homogeneous irradiation.

A, scale = 10 cm.; B, anticathode; C, lead screen with 3 mm. slit; D, lead screen with 2 mm. slit; E, lead screen with 2 mm. slit; F, mica; G, screw for moving E; H, experimental box.

(c) *Scattered Heterogeneous Beam*.—The intensity of the homogeneous beam was several hundred times less than that of the direct heterogeneous beam, and in order to obtain a more direct comparison of the biological action produced by the two types of beam, it was necessary to obtain a heterogeneous radiation of the same order of intensity as that of the homogeneous radiation. A heterogeneous beam of the desired intensity was obtained from the rays scattered when the direct beam from the X-ray tube penetrated a block of hard paraffin. The arrangement of the apparatus is illustrated in fig. 4. The scattered

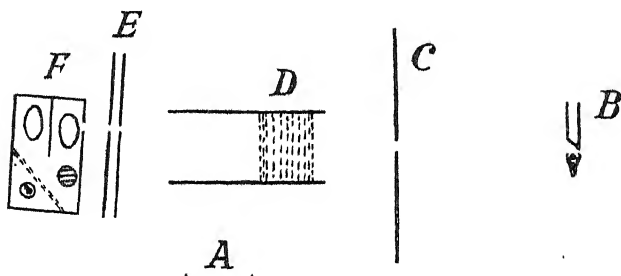


FIG. 4.—Apparatus for weak heterogeneous irradiation.

A, scale = 10 cm.; B, anticathode; C, lead screen with 3 mm. slit; D, paraffin contained in lead sleeve; E, 2 lead screens each with 2 mm. slit; F, experimental box.

radiations were allowed to pass through two vertical slits 2 mm. wide before reaching the exposed egg, so that the rays used were approximately parallel. The position of the slits and the thickness of the paraffin block were adjusted

so that the intensity of the scattered beam which reached the egg was exactly the same as the intensity of the homogeneous beam which has been described.

A scattered radiation is of the same constitution as the primary beam which excites it, and the energies associated with each wave-length are in the same proportions in both the scattered and the exciting beams (Kaye, 1926, p. 113). In these experiments therefore the scattered and the direct beams of X-rays differed only in intensity.

Measurement of Intensity.

A gold-leaf electroscope of the usual pattern was used for the measurement of intensity, and by means of this instrument the intensity of the primary beam, which was produced in all experiments at a kilovoltage of 78 and a milliamperage of 5, was found to be constant. The mass absorption coefficient for aluminium of the primary beam was measured and found to be 0.24. Owing to lack of apparatus it was not possible to measure the intensity directly in r units, but the absolute intensity was measured approximately by Holznacht pastilles. One skin erythema dose or 5 H units was equal to an exposure of 30 minutes at the standard distance of these experiments. The skin erythema dose at a kilovoltage of 78 is approximately equal to 450 r units (Knox, 1932, p. 65), and therefore one minute's exposure to the primary beam was approximately equal to 15 r units. For convenience of comparison the doses are recorded here as r units, but these doses are only approximate although the relationship between the different doses was measured accurately.

The intensity of the primary X-ray beam was enormously greater than that of the reflected homogeneous beam, and the two intensities were compared in the following way. The rate at which the reflected homogeneous beam discharged the electroscope was measured and the crystal was then removed so that the primary beam reached the electroscope directly through the same slit as was used for the homogeneous beam. Filters of aluminium (about 5 cm.) were interposed in the path of the primary beam until the rate of discharge of the electroscope was exactly that which was produced by the reflected homogeneous beam. The intensities of the filtered ray and of the primary beam therefore bore the same relationship to each other as did the intensities of the reflected ray and of the primary beam.

The electroscope was moved to a distance of about 180 cm. from the anticathode, and the unfiltered primary beam was allowed to reach it through a very small slit of known area— S_1 . The rate of discharge of the electroscope was measured. The primary beam was again filtered with the same thickness

of aluminium which had previously been used. The slit was increased in size and adjusted until the filtered and unfiltered beams produced the same rate of discharge, and the area of the enlarged slit was measured $-S_2$. The intensities of the filtered and of the unfiltered beam bore the relationship to each other of S_1 to S_2 , and this relationship was the same as that of the intensity of the homogeneous beam to that of the primary. It was thus found that the intensity of the homogeneous beam was $1/937$ of the intensity of the primary beam.

Preparation of the Eggs.

Hens' eggs were incubated for 9 days, during which time the allantois had grown almost completely round the internal surface of the shell. By means of a viewing box an area of allantoic membrane, situated some distance away from the embryonic stalk, was chosen, in which the blood vessels were of medium size. Over this area a window 1 cm. long and 0.5 cm. wide was marked out on the shell with the long axis of the window in the long axis of the egg. The shell was rapidly cleaned with rectified spirit and the window sawn out. The rectangular piece of shell was carefully detached from the underlying shell membrane in one or two pieces. This shell membrane is a

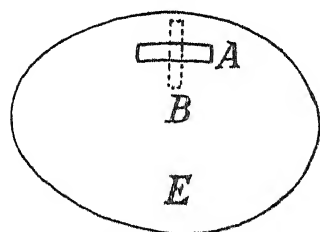


FIG. 5.—Showing position on egg of egg window and irradiated area.

E, egg; A, continuous line represents position of shell window; B, dotted line represents area over which X-rays fell.

is adherent to it, and separates the underlying allantois from the shell itself. Those eggs were discarded in which this fibro-elastic membrane was broken or in which any damage to the allantoic membrane was suspected. The egg so treated was placed in the experimental box with the egg window opposite the box slit so that the narrow X-ray beam crossed the centre of the shell window, fig. 5. At the end of the exposure the removed piece of shell was cleaned with rectified spirit and replaced, the whole window being sealed with adhesive plaster.

The egg was returned to the incubator for 4 days, and was then fixed in "Susa" solution. Sections were made through that part of the allantois which lay adjacent to the egg window and were cut parallel to the long axis of the window.

The experiments involved the exposure for several hours of egg membranes deprived of their shell covering. It was therefore of great importance to

prevent injury by drying or cooling and also to make rigorous controls. The precautions adopted were as follows:

During their irradiation the eggs were contained in a box diagrammatically represented in fig. 6. The box was of lead with a close fitting lead cover and was heated by an electric lamp. The only opening was a vertical slit 1 cm. long and 0.5 cm. wide, through which the X-rays passed, and this was covered with gold-beaters' skin to prevent passage of air currents to and from the interior of the box. Two positions for eggs are shown in the diagram, fig. 6, separated from each other by a lead screen; that next to the window was the position for the experimental egg, and the other that of the control egg. The interior of the box was kept saturated with water vapour at a temperature of 39° C. Thus the conditions in the box were virtually the same as in an incubator.

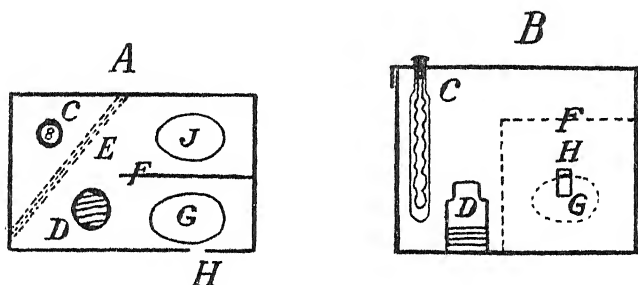


FIG. 6.—Experimental box.

A, plan of box; B, elevation; C, heating; D, water; E, asbestos screen; F, lead screen to protect control egg; G, experimental egg; H, window of box; J, control egg.

This technique in relation to eggs was employed throughout the whole course of this investigation except in those experiments in which a modified technique described later, was used.

Response of the Allantois.

The allantois in my experiments showed two types of response to stimuli:—

(1) Hyperplasia, and (2) Atrophy.

Figs. 7, 8 and 9, Plate 16, show typical photomicrographs of these two effects. They resemble exactly the results described by Moppett (1929) and by Goulston and Mottram (1932). These authors have described the conditions in detail and a repetition of their descriptions is unnecessary.

The atrophic response when present was always unmistakable. Hyperplasia when fully developed was equally unmistakable, but sometimes minor degrees of hyperplasia occurred which could not be distinguished with certainty

from the normal. For this reason chief attention was directed to the atrophic response, and I endeavoured to estimate the dose needed to produce atrophy in 50 per cent. of experiments (median atrophic dose).

Control Experiments.

Experiments were done in order to find whether the simple procedure of removing a piece of shell and leaving the shell membrane exposed for a period could produce visible changes in the allantois. Eggs treated in the way described were left in the experimental box for varying periods without any further interference. At the end of these periods the windows were closed and the eggs were reincubated. In Moppett's and in my experiments the longest times of exposure to irradiation were 180 minutes, whilst many exposures were of 120 minutes. These exposures were therefore used in the control experiments. Fig. 6 shows the position of the control egg during the exposure of the experimental egg to X-rays and the results obtained in 42 such experiments are shown in Table I.

Table I.—Control Experiments.
Effect of Exposure of Egg Window for 2–3 hours.

Result.	Number of eggs.	Percentage.
No change	33	79
Hyperplasia	9	21
Atrophy	0	0

During the progress of these experiments certain criticisms of Moppett's method were published by Goulston and Mottram (1932), one of which pointed out that the control egg was not adjacent to the window through which the X-rays passed, and therefore was not subjected to currents of air which such a window might occasion. Sixteen consecutive control experiments were therefore carried out in which the eggs were in contact with the window, and hence the conditions were exactly comparable to those in irradiation experiments. The results which are recorded in Table II are identical with those obtained with the larger number of controls (Table I).

Table II.—Special Control Experiments.
Effect of Exposure of Egg Window for 2–3 hours.

Result.	Number of eggs.	Percentage.
No change	13	81
Hyperplasia	3	19
Atrophy	0	0

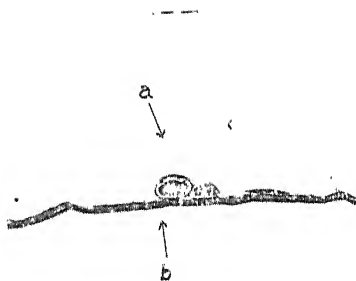


FIG. 7.

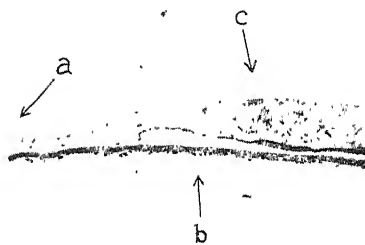


FIG. 8.

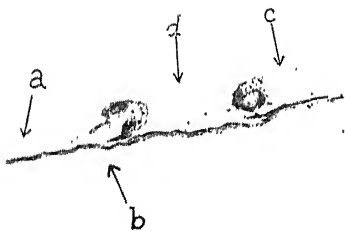


FIG. 9.

The results show that exposure for 2-3 hours never produces atrophy but does produce hyperplasia in 10-20 per cent. of cases. Since such exposures did produce some measurable effect, the effects produced by a wide variety of durations of exposure were examined. These results are shown in Table III. The mere opening of the shell produced no effect, but exposures for periods of 6 hours or more produced atrophy in a large proportion of cases. The figures for the long exposures are scanty because in the majority of cases the long exposures caused death of the embryo within 4 days, and the local effects could therefore be determined only in a minority of cases.

Table III. -Effect of Various Exposures of the Egg Window to Air.

Duration of exposure.	No. of experiments.	No change.	Hyperplasia.	Atrophy.	Percentage of atrophy.
Less than 1 minute	6	6	0	0	0
2 to 3 hours	58	46	12	0	0
6 hours	7	0	4	3	43
9 hours	5	0	0	5	100

Table IV summarises the results obtained by Moppett (1929), by Goulston and Mottram (1932) and by me, and it will be seen that my results are intermediate between the others. Moppett thought that exposure alone produced

Table IV.—Comparison of Results of Control Experiments with less than 3 hours' exposure to Air of the Shell Window.

Author.	No. of experiments.	No change.	Hyperplasia.	Atrophy.	Percentage of atrophy.
Moppett.....	30++	30++	0	0	0
Goulston and Mottram	13	5	7	1	8
Scott	58	46	12	0	0

no effect on the allantois, whilst Goulston and Mottram found that exposure alone usually produced hyperplasia and sometimes produced atrophy. My results show that exposure for 2-3 hours does not produce atrophy but sometimes produces hyperplasia. Table III shows that more prolonged exposures can undoubtedly produce atrophy. It was also found that mechanical injury of the shell membrane easily produced atrophy.

The results of Goulston and Mottram (1932) suggest obvious doubts as to the validity of conclusions drawn from the effects produced by irradiation associated

with long exposure of the shell membrane to air. My results, however, show that exposure for periods not exceeding 3 hours does not produce atrophy and only occasionally produces hyperplasia. In my experiments, therefore, the occurrence of atrophy or the occurrence of a high percentage of hyperplasia after exposure to X-rays for periods of not more than 3 hours must be attributed to the action of X-rays and cannot be accounted for by exposure to air alone. On the other hand, there is no doubt that exposure alone injures the egg membranes, and hence very few deductions can be drawn from differences between the results produced by irradiation of intact eggs and those produced by irradiation of eggs with shell windows.

Effect of Irradiation of Intact Eggs.

Eggs were treated in the usual way except that no window was cut. They were irradiated by the direct beam from the X-ray tube over a small marked area; the arrangement of the apparatus is shown in fig. 1.

Strangeways and Fell (1928) irradiated the whole of hens' eggs with X-rays on the sixth day of incubation and found that 150 *e* units (1 *e* unit = 3.4 *r* units [Knox, 1932, p. 62]) caused the death of the embryo during the subsequent 24 hours of incubation. Eggs at the 17th day of incubation were killed by 270 *e* units.

I found that four times this dose, 3000 *r* units, produced no effect on the allantois. The lethal action of such doses was reduced as low as possible by directing the X-ray beam almost tangentially to the egg surface so that the embryo escaped the direct rays. Even with these precautions a dose of 3000 *r* units killed 30 out of 36 eggs, but the six survivors showed no change in the allantois.

This negative result cannot be ascribed to absorption of the rays by the shell, for the absorption of 12 different egg shells was measured and found in all cases to be less than 20 per cent.

Direct Irradiation.

Eggs with the usual shell window were subjected to the direct rays from the X-ray tube, fig. 1. The results obtained are shown in Table V, and it will be seen that the median atrophic dose is about 700 *r* units. This dose was given in less than an hour, and therefore the atrophy observed must have been due to an effect produced by the X-rays since the controls showed no atrophy after exposure to air for 3 hours (Tables I and II). Furthermore, the atrophy

always occurred in that position of the exposed membrane which was subjected to the X-ray beam. The results recorded by Moppett (1929) appear to agree with the results shown in Table V.

Table V. --Direct Irradiation.

X-ray dosage approximate in r units.	Exposure in minutes.	Number of eggs.	No change.	Hyper- plasia.	Atrophy.	Percentage of atrophy.
75-300	5-20	3	3	0	0	0
450-700	30-45	5	1	3	1	20
700-900	50-60	5	0	1	4	80

Homogeneous Irradiation.

The method of the production of the homogeneous beam of X-rays has already been described, and the arrangement of apparatus is shown diagrammatically in fig. 3. The wave-length of the homogeneous beam was 0.2 Å, and the intensity was 1/1000 of that of the direct beam.

Eggs with windows which were prepared in the usual way were irradiated with the homogeneous X-rays which reached the egg as a rectangular pencil of rays with the long axis of the rectangle at right angles to the shell window, in the manner which has been described in the case of the direct mixed beam, fig. 5.

The results, which are given in Table VI, show that the median atrophic dose of the homogeneous beam is about 2 r units. The exposure to air in this case was not more than 180 minutes, and therefore, judged by the same criteria as those applied in the case of direct irradiation, the atrophy here recorded must have been a result of X-ray injury.

Table VI. --Homogeneous Irradiation.

Approximate dose in r units.	Exposure in minutes.	Number of eggs.	No change.	Hyper- plasia.	Atrophy.	Percentage of atrophy.
0.5	20-35	3	3	0	0	0
0.5-1.0	35-60	5	2	3	0	0
1-2	60-100	7	2	4	1	14
2-3	100-180	7	0	2	5	71

The median atrophic dose of direct mixed irradiation, however, was about 700 r units, that is to say the relationship between doses of mixed and dosages of homogeneous radiations which produced the same biological action was in

the ratio of about 700 to 2 under the conditions of these particular experiments. My results therefore agree with those of Moppett in showing an apparently extraordinary difference in the biological action of the two types of X-rays. But these remarkable results do not directly prove a difference in the biological activity of homogeneous and of heterogeneous X-rays because there was an important difference between the duration of exposure of the egg window to air in the two series of experiments. The administration of 700 *r* of direct mixed irradiation took about an hour, whereas the administration of a dose of 2-3 *r* of homogeneous irradiation took 2-3 hours.

Exposure to air undoubtedly influenced the response of the allantois to X-rays, for a dose of about 3000 *r* failed to produce atrophy in unopened eggs, whereas 700 *r* produced this effect in eggs which had been opened and exposed for an hour. Exposure without irradiation for periods up to 180 minutes never produced atrophy, but occasionally produced hyperplasia. Genuine atrophy, however, was produced by exposure for 6 hours when no irradiation was given. These results suggest that exposure alone for 180 minutes produces about half the injury needed to cause atrophy and that the effects of exposure and radiation are additive.

Experiments were therefore carried out in which a weak heterogeneous beam was used and the time of exposure of the egg window to air was the same as the time of exposure in the experiments with homogeneous irradiation.

Scattered Heterogeneous Irradiation.

Eggs with the usual egg window were irradiated with a weak heterogeneous beam of X-rays which was scattered from a block of hard paraffin, fig. 4. The intensity of this beam was exactly the same as that of the homogeneous beam used in the preceding series of experiments.

The results which are recorded in Table VII show that a dose of 2-3 *r* units of weak heterogeneous irradiation, administered over a period of 100-180

Table VII.—Scattered Heterogeneous Irradiation.

Approximate dose in <i>r</i> units.	Duration of irradiation in minutes.	Number of eggs.	No change.	Hyperplasia.	Atrophy.	Percentage of atrophy.
0.5-1	15-40	3	3	0	0	0
1-2	40-100	6	2	2	2	33
2-3	100-180	9	0	4	5	56

minutes, produced atrophy in about 50 per cent. of cases. The same result was produced by a weak homogeneous beam when 2-3 r units were applied over a similar period. The exposure of the egg window to air was therefore the same in both series of experiments. In the two series similar doses of X-rays of similar intensity produced identical biological effects although one beam was homogeneous and the other mixed.

This result shows that homogeneous and mixed beams act in an identical manner on the allantois of the hen's egg, and it cannot be reconciled with the hypothesis put forward by Moppett that a homogeneous beam of X-rays has an enormously greater biological action than a heterogeneous beam, and that X-rays of different wave-lengths antagonise each other in their biological effects.

There remains to be explained, however, the remarkable contrast between the biological effects produced by weak beams of X-rays, whether homogeneous or mixed, and those produced by intense beams. The median atrophic response of the allantois to weak irradiation is produced by 2-3 r units applied over a period of 100-180 minutes, whereas the same response to intense irradiation is produced by 700-900 r units applied over a period of 40-60 minutes. Now in these two types of experiment there were three factors which varied :-

- (a) The dose of X-rays.
 - (b) The length of time which was required to administer the dose of X-rays.
 - (c) The length of time during which the egg window was exposed to air.
- Experiments were therefore devised in order to determine the relative importance of these three variable factors.

Short Period of Irradiation and Long Period of Exposure of Egg Window to Air.

Since weak irradiation, both homogeneous and heterogeneous, produced atrophy in over 50 per cent. of experiments when the egg window was exposed to air for 100-180 minutes, experiments were done in which an exposure to air for this length of time was combined with direct irradiation from the X-ray tube, administered over a very short period. The dose of X-rays administered was small, namely, 1.5 to 7.5 r .

Eggs were incubated for 9 days and a shell window was cut. The experimental egg was exposed in the box for times varying from 100-180 minutes. Towards the end of that period the egg was irradiated over the shell window by the direct radiation from the X-ray tube for periods up to 0.5 minute. The

shell window was replaced immediately and the egg reincubated for 4 days when sections were cut. The results are recorded in Table VIII.

Table VIII.—Effect of Exposure to Air for 2–3 hours followed by Immediate Irradiation for Short Periods.

Dose in r units.	Duration of irradiation in minutes.	Number of eggs.	No change.	Hyper- plasia.	Atrophy.	Percentage of atrophy.
0.4	0.03	3	1	2	0	0
1.5	0.1	5	0	3	2	40
3.0	0.2	10	2	4	4	40
7.5	0.5	6	1	1	3	50

Similar experiments were done in which the allantois was irradiated immediately after the cutting of the shell window and the exposure to air for 100–180 minutes was consecutive. These results are recorded in Table IX.

Table IX.—Effect of Exposure to Air for 2–3 hours immediately preceded by Irradiation for Short Periods.

Dose in r units.	Duration of irradiation in minutes.	Number of eggs.	No change.	Hyper- plasia.	Atrophy.	Percentage of atrophy.
4.5	0.3	3	0	1	2	66
7.5	0.5	3	0	0	3	100

The results recorded in Tables VIII and IX show that a dose of X-rays between 1.5 and 7.5 r units administered within 1 minute in the form of an intense mixed beam can produce atrophy of the allantois in 50 per cent. of experiments provided that the shell window over the irradiated area is exposed to air for a period of about 100–180 minutes. This dose of X-rays is of the same order of magnitude as those which were used in the experiments with reflected homogeneous and scattered mixed radiations where the intensity of the X-rays was very small. Provided therefore that the egg membranes are exposed to air for a certain time (2–3 hours) the same effects are produced by the same dose of X-rays whether the radiation is intense and short (0.5 minute), or weak and prolonged (2–3 hours). The results in the latter case cannot therefore be attributed to any deviation from the Bunsen-Roscoe law. They indicate, however, that the injurious effects of irradiation and of exposure to air are additive.

Effect on the Allantois of Two Additive Stimuli applied at 24 hours' Interval.

It has been shown that irradiation of the intact egg with very large doses of X-rays produces no visible changes in the allantois. It has also been shown that exposure of the egg window to air for periods up to 180 minutes never produces atrophy. Since atrophy occurs when these two stimuli are added it appears that each stimulus produces some change in the allantois, and a few experiments were carried out to find whether these changes are reversible.

Intact eggs were irradiated over a marked area with the direct beam from the X-ray tube for periods up to 0.5 minute. The eggs were reincubated for 24 hours. A shell window was then cut over the marked area, and this window was exposed to air for about 120 minutes. The allantois was sectioned 4 days later. Three control experiments were done which differed only in the duration of the exposure of the window to air, which was in their case only 5 minutes.

In a second type of similar experiment the order of irradiation and exposure to air were reversed. A shell window was cut and was exposed to air for about 150 minutes. The shell was replaced and the window was sealed with thin strips of adhesive plaster around the window margin. The egg was reincubated for 24 hours and the allantois was irradiated through the closed window. The egg was reincubated for a further 4 days and the allantois sectioned.

The results, which are recorded in Table X, show that atrophy of the allantois occurs when a dose of X-rays of 4-8 *r* units is applied through the shell, followed or preceded at 24-hours interval by an exposure of the allantois to air for about 150 minutes. Tables VIII and IX showed that the same dosage of X-rays combined with the same duration of exposure of the egg window to air produces the same result when there is no interval between the time of irradiation and

Table X.—Effect of Irradiation and Exposure to Air separated from each other by an interval of 24 hours.

Type of experiment.	Approximate dose in <i>r</i> units.	Duration of irradiation in minutes.	Number of eggs.	Duration of exposure of shell window to air in minutes.	Result.
A	7.5	0.5	3	5	No change.
B	4.5-7.5	0.3-0.5	4	120-135	2 hyperplasia, 2 atrophy.
C	6	0.4	3	150	1 hyperplasia, 2 atrophy.

A.—Irradiation of intact egg with short exposure of the shell window to air 24 hours later.

B.—Irradiation of intact egg with long exposure of the shell window to air 24 hours later.

C.—Long exposure of the shell window to air with irradiation through the shell 24 hours later.

the time of exposure of the egg window. It is therefore concluded that the processes set up in the allantois by these doses of X-rays and these exposures to air are more or less irreversible in 24 hours. These results also show that irradiation of the egg through the shell does produce a change in the underlying allantois, although this change cannot be demonstrated unless the allantois is injured by exposure to air.

Discussion.

The experiments which have been described show that an extremely small dose of X-rays administered in the form of a beam of very feeble intensity can cause the complete destruction of living tissue. The significance of the experiments with reflected homogeneous and scattered heterogeneous beams of X-rays on which this conclusion is based, obviously depends on the complete reliability of control experiments. These have been fully discussed, and they have shown that while atrophy of the allantois of hens' eggs occurs after irradiation with 2-3 *r* units under certain conditions, those conditions alone without irradiation never produce atrophy.

Thus the results of Moppett's experiments with a homogeneous beam have been confirmed, but his conclusion, that there is a remarkable difference in the biological action of homogeneous and heterogeneous beams, cannot be supported. The effects of weak homogeneous and equally weak heterogeneous X-rays, Tables VI and VII, were identical, and these effects were produced under exactly similar conditions. Homogeneous and heterogeneous beams of X-rays therefore produce identical effects on the allantois of hens' eggs, and since the same doses of both types of ray produce the same result, there can in this case be no antagonism between the different components of the heterogeneous beam.

It is necessary, however, to account for the result, obtained by Moppett and confirmed by me, that a dose of 2-3 *r* units administered over a period of 2-3 hours produced the same effect as a dose of 800 *r* units administered in 1 hour. In the two types of experiment the duration of the exposure of the shell window to air was different, and the explanation may lie either in this difference or in a deviation from the Bunsen-Roscoe law. The various combinations of X-ray dosage and duration of exposure to air are summarised in Table XI. This table shows that a dose of 2-3 *r* units combined with exposure to air for 2-3 hours produces the same effect whether the radiation is given in less than 1 minute by a relatively intense irradiation or is spread over 2-3 hours in the

form of a reflected beam either mixed or homogeneous in quality. In these experiments, therefore, the Bunsen-Roscoe law holds.

Table XI.—Combinations of Exposures to Air and Irradiation by X-rays which produce Atrophy in about 50 per cent. of Eggs.

Quality.	Irradiation.		Duration of exposure to air in minutes.
	Duration of irradiation in minutes.	Dose in r units.	
<div style="display: flex; align-items: center;"> <div style="margin-right: 10px;"> Feeble homogeneous Feeble heterogeneous </div> <div style="font-size: 2em; margin-right: 10px;">}</div> <div> Nil with simultaneous exposure to air </div> </div>	Nil 120-180	Nil 2-3	400 120-180
<div style="display: flex; align-items: center;"> <div style="margin-right: 10px;"> Direct heterogeneous immediately followed by exposure to air. Direct heterogeneous immediately after exposure to air. Direct heterogeneous with 24 hours interval between irradiation and exposure to air. </div> <div style="font-size: 3em; margin-right: 10px;">}</div> <div> 0.1-0.5 </div> </div>	0.1-0.5	1.5-7.5	120-180
Direct heterogeneous with simultaneous exposure to air	50-60	700-900	50-60
Direct heterogeneous irradiation of the intact egg	—	>3000	Nil

It has already been shown that exposure to air alone injures the allantois, and Table XI shows that shorter exposures to air, one hour, need large doses of X-rays to produce the median atrophic response, while longer exposures, 2 hours, need only a very small dose of X-rays. The injury which is produced in the allantois is therefore the result of both X-rays and exposure to air, and the effects of these two agents appear to be in some way additive. No simple relationship, however, exists between them for 1 hour's exposure to air combined with a dose of 700-900 r produces a median atrophic response, but if the duration of the air exposure be doubled then a dose of only 2 r is required to produce the same effect. The effect of exposure of the shell window to air appears to potentiate the action of X-rays rather than to superimpose an additional injury on that produced by the rays.

This potentiating action occurs not only when the allantois is irradiated with a weak beam during the whole period of its exposure to air, but also when the irradiation is intense and is given either at the end or the beginning of the exposure. The action occurs not only when the irradiation is applied 24 hours after the exposure to air, but also when it is applied 24 hours before the exposure.

These observations offer no explanation of the nature of the potentiation, but they suggest that whatever injury is produced either by X-rays or by exposure of the allantois to air, that injury is repaired either very slowly or not at all.

The reactions of the allantois are summarized in the following way. Atrophy occurs when the allantois is exposed to air for 6 hours without irradiation. The uninjured allantois is very resistant to X-ray injury, but exposure to air for 2-3 hours alters it so that it becomes extremely sensitive to the action of X-rays.

The use of the allantois of the hen's egg for an investigation of the nature of the biological action of X-rays therefore involves the irradiation of damaged tissue. The nature of the damage is unknown, and its measurement by duration of the exposure of the allantois to air is obviously so crude that it is considered that the allantois is not an ideal preparation for the study of the action of X-rays and, least of all, for the provision of quantitative results.

Acknowledgments.

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Summary.

(1) An extremely small dose of X-rays, 2-3 *r* units, can cause the death of living tissue.

(2) Homogeneous and heterogeneous X-rays have an identical biological action on the allantois of the chick.

(3) There is no antagonism between the components of different wavelengths of a heterogeneous beam of X-rays in their action on the allantois of the chick.

(4) The variation of intensity of X-rays used when administering a certain dose of radiation makes no difference to the resultant biological action, *i.e.*, the Bunsen-Roscoe law is true in these experiments.

(5) The effects of the exposure to air of a shell window in a hen's egg potentiates the action of X-rays on the underlying allantois.

(6) These effects on the allantois are irreversible in 24 hours.

(7) The allantois of the chick is not an ideal preparation for the study of the biological action of X-rays.

Note.—Since the preparation of this paper Goulston (1932) has described two experiments in which the allantois was irradiated by homogeneous rays with negative results. A technique similar to that which has been described was used, but the egg window was opened under completely aseptic conditions. The dose of homogeneous irradiation was not stated.

DESCRIPTION OF PLATE 16.

(a) Normal allantois ; (b) shell membrane ; (c) hyperplasic allantois ; (d) area of atrophy.

FIG. 7.—The normal allantois ($\times 13$).

FIG. 8.—The hyperplasic response ($\times 13$).

FIG. 9.—The atrophic response ($\times 13$).

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The Principles of Ultrafiltration as Applied in Biological Studies.

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[PLATE 17.]

Ultrafiltration methods have a twofold usefulness :—(1) As a general means of fractionating disperse systems, and (2) in providing data enabling the size of dispersed particles to be estimated. Their successful application requires an understanding of the physical processes involved. An ultrafilter membrane does not behave like an ordinary sieve in which coarseness of mesh alone determines whether or not a non-deformable particle shall pass. As the meshes become smaller the relative importance of the thickness of the sieve increases, until eventually, as in the case of a membrane, the length of a pore becomes very much greater than its diameter. The liquid traversing the pores of a membrane is in contact with a very large surface, and hence surface phenomena may be expected to play an important part in an ultrafiltration process. The ratio, area of pore surface/pore volume, varies inversely with the first power of the pore radius and hence becomes very large for ultrafine capillaries.

Before proceeding to consider experimental evidence upon the course of filtration with typical disperse systems under varied conditions, it will be well to form a working conception of the structure in ultrafilter membranes. Collodion films which are the most commonly used ultrafilters have been studied from the point of view of their structure (Elford, 1930), and the evidence of optical examination indicated an arrangement of aggregated particles somewhat analogous to a pile of shot. This implies lateral as well as vertical permeability, while the degree of porosity will be determined by the size of the particulate units and their arrangement and closeness of packing in successive planes. The analogy cannot be regarded as in any sense a strict one, however, since the colloidal nitrocellulose particles are not spherical, appearing in the ultramicroscope to be slightly elongated, while their mutual orientation is determined by fields of molecular forces that are strongly polar in nature. The effective channels through such membranes approximate to long capillaries, and since the flow of liquid through the membrane will occur

most readily by the shortest path from face to face, these capillaries are relatively straight. Evidence may be cited in support of this. Poiseuille's law is found to govern the flow of water through the membranes over ranges of low pressure where no distension of the membrane occurs; also when a dye suspension is filtered through a membrane supported upon a perforated plate, the circular areas, through the pores of which the dye passes, become deeply stained through the entire membrane thickness. The corresponding stained circular areas visible on the under face of the membrane remain sharply defined even after long periods of filtration. Lateral diffusion between the mainly effective capillaries therefore occurs very slowly. Thus the ultrafilter membrane may be regarded from the point of view of its performance as a porous structure, the effective pores being long and relatively straight channels, formed by the communicating interstices, between the elements in superimposed strata of the aggregated nitrocellulose particles.

The Mechanism of Filtration.

In attempting to form a general conception as to what will happen when a dispersoidal solution is filtered through a membrane of given porosity, consideration must be given to three main factors:—

- (i) Surface equilibria between the substance of the membrane and the disperse phase of the solution, owing to surface adsorption and cohesion.
- (ii) Reaction between the filter septum and the continuous phase of the solution, causing imbibition or swelling of the membrane substance.
- (iii) Internal equilibria in the dispersoid solution, involving solvation of the disperse phase, degree of aggregation in relation to the electrolytic content, viscosity.

Of these (i) and (iii) are of primary importance in practical ultrafiltration. The collodion membrane, equilibrated in water, is very stable in contact with aqueous solutions; but in diffusion and filtration processes in living membranes, sensitive to delicate changes in the reaction and salt content of the fluids permeating them, the phenomenon of imbibition undoubtedly plays a prominent rôle.

In the filtration of a colloidal solution, therefore, we are concerned with what is essentially a two-stage process. Stage 1 involves the adjustment of equilibrium between the surface of the capillaries and the solution, while stage 2 consists of true filtration through the modified, equilibrated membrane. Typical filtration curves obtained by plotting the concentration of disperse

phase contained in the filtrate against the total volume of filtrate are given in fig. 1. The filtrate concentration is expressed as a percentage of the initial concentration. The interpretation of these curves is perfectly straightforward in terms of the ordinary laws of surface equilibria (Freundlich, Langmuir, Gibbs-Thomson). Thus curve I represents the course of filtration in the case

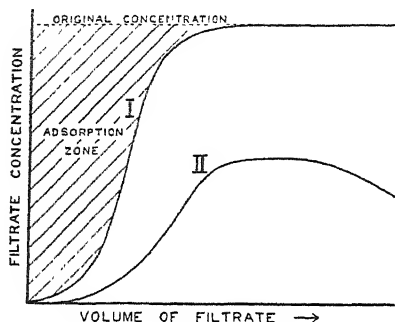


FIG. 1.

of a sol of which the particles are sufficiently small to pass the filter readily. As Zsigmondy (1909) has pointed out, in explaining his experience with various types of filter candle, the filter surface has a certain adsorbing capacity, and this applies also to the case of membranes. This affinity must first be satisfied before unhindered passage through the filter occurs. Hence on the curve shown, the filtrate is at first poor in the disperse

phase but, when once surface equilibrium is attained, the concentration of the disperse phase in the filtrate rapidly reaches that of the original unfiltered solution.

For a polydisperse system, in which a fraction of the disperse phase is too gross to pass the pores of the filter, the course of filtration is shown in curve II. The filtrate concentration never reaches that of the original solution, but attains some maximum partial value and then begins to fall off again, as the concentration of unfilterable particles on the surface of the filter progressively reduces the effective permeability, and the membrane becomes mechanically blocked.

A third condition occurs when the initial porosity, or the effective equilibrium porosity, of the membrane is such as to prevent completely the passage of the disperse phase. Pure solvent or continuous medium only appears in the filtrate. The membrane is, in fact, semi-permeable.

The internal equilibria of the disperse system itself, as reflected in viscosity phenomena, are very important in filtration. While viscosity in a liquid in bulk may be a perfectly characteristic quantity, it is to be expected that, in considering flow in capillary tubes, a critical value in capillary dimensions will be reached, where a departure from the laws of true viscous flow will be observed. The zone next the pore wall, where the molecules come under the influence of surface forces, is no longer negligible in its effect upon the flow. With colloidal systems departures from the linear relationship between

rate of flow and shearing stress are met with in macroscopic capillaries, and represent a peculiar property of the system itself. Viscosity phenomena are intimately associated with the size and shape of the molecular and colloidal aggregates, and to explain the departure in colloidal systems from the linear relationship above mentioned McBain, Harvey and Smith (1926) have postulated the existence of structures of "ramifying aggregates," which yield under increased shearing stress. The tendency to structural association is manifested in a limiting condition by spontaneous gelation. It is not surprising, therefore, to find colloids which are exceedingly difficult to filter owing to their rapid blocking of the filter—for example, the solution of hæmoglobin from freshly washed red cells laked in distilled water.

The stability of a disperse system is accordingly another factor of importance in filtration. One of the chief factors in initiating a coagulating process is the chance of collision between the disperse particles. In bulk this may be sufficiently low to conserve apparent stability, but, when the system is forced through a long narrow channel, the chances of collision are increased many-fold. Hence coagulating processes may be precipitated by filtration, and systems whose non-filterability arises from such a cause may often be rendered filterable by incorporating a protective agent. Thus Zsigmondy (*loc. cit.*) has found that egg albumin facilitates the passage of gold sol particles ($30\ \mu\mu$) through filter candles.

Experimental.

Unless otherwise specifically stated, the collodion membranes used in the experimental studies have been the new series of graded membranes, described by the author in an earlier paper (1931) in which the method used in estimating the average pore diameter (A.P.D.) is also given. The preparation of these membranes, with the mixtures of collodion solvents and other details of treatment necessary for the regular reproduction of the different grades of porosity, have been fully described in the earlier communication. For convenience of reference they were called "Gradocol" membranes, by which name they are further mentioned here.

The design of the ultrafilter has been given previously (Barnard and Elford, 1931). Positive applied pressures of air or nitrogen have been employed in filtering the liquids, and the course of filtration has been followed by collecting successive samples of filtrates, and subjecting these, and, in some instances, the residue also, to analysis. The curve of filtration for each system may then be constructed.

External Filtration Conditions and their Influence upon the Process of Filtration.

The resultant effect on filtration of varying such factors as the concentration of the suspension, the volume of filtrate collected, the thickness of membrane, area of membrane surface, and the filtration pressure can be conveniently studied with dye solutions. Estimations of dye concentrations may be made by colorimetric methods, while the dyes also provide a range of sols with positive and negative electric charges which enable adsorption phenomena to be investigated. The importance of adsorption in filtration has long been recognized. Bechhold (1920) has emphasized the advisability of testing the adsorptive properties of the filter membrane by shaking fragments of it with the solution, and investigating the latter afterwards. A qualitative test of this kind, however, does not enable the influence of adsorption in the actual filtration process to be quantitatively assessed. This can only be done by establishing filtration curves and studying how these are modified by changing conditions.

Effect of Concentration.—It has been found in experiments with dye solutions of different concentrations, using comparable filtration conditions, that the amount of filtrate which has to be collected before the maximum concentration of solute is attained in the filtrate, depends upon the concentration of the initial solution. Over a tenfold variation in initial concentration the relationship is one of slightly less than inverse proportionality (see Table I).

Influence of Membrane Thickness and Area.—Increase in the membrane thickness or area increases the adsorbing surface proportionally. Experiment has shown that, under otherwise comparable conditions, the adsorption in filtration is proportional to the membrane thickness, and also to the membrane surface area. At low pressures, however, the apparent adsorption is somewhat more than directly proportional to the membrane thickness, presumably owing to the greater scope offered to cohesive forces under slow rates of filtration.

Effect of Applied Pressure.—In instances where the initial adsorption stage of the filtration process is of minimal and even negligible proportions, the effect of increasing the filtration pressure is simply to increase the rate of filtration. When the effects of surface forces are appreciable, however, the influence of pressure may be very marked in the early stages of filtration, particularly over a range of permeability immediately preceding the end-point for the disperse phase. The curves in fig. 2 are from data obtained by filtering a 0.01 per cent. aqueous solution of night blue (previously filtered through a

Table I.

Total volume of filtrate, c.c.	0.25	0.5	1	2	3	4	5	6	7	8	10	22.5	25	30	35	Remarks.
	Concentration of dye in filtrate.															
Initial dye concentration per cent.																
0.5	0	+	0.02 4	0.4 80												Membrane grade = 0.4 μ . Thickness = 0.1 mm. Filtration pressure 30 cm. H ₂ O.
	0	0	0	0.025 25	0.075 75	0.1 100										
0.05	0	0	0	0	0	0	+	0.006 12	0.0125 25	0.04 80	0.05 100					
0.01	0	0	0	0	0	0	0	0	0	0	0	+	0.0025 25	0.006 60	0.008 80	

Note.—The upper figure in the filtrate concentration column is the actual per cent. concentration of dye; the lower figure expresses this concentration as per cent. of the original.

0.6 μ membrane to remove coarse particles) through 0.25 μ membranes under different applied pressures. It is manifest that as the pressure is increased the adsorption zone becomes compressed, and that the slope of the curve also becomes steeper. This effect is doubtless partially explained by the fact that as the pressure is increased the precise area of membrane surface becomes more strictly confined to the areas corresponding to the perforations in the supporting plate, as is indicated by the enhanced sharpness of definition in the stained areas on the under face of the membrane. Even so, as might be anticipated, a tendency towards a minimum adsorption characteristic of the membrane is suggested. This probably corresponds to the primary adsorbed layer, the influence of pressure being in respect of secondary diffuse layers, where the

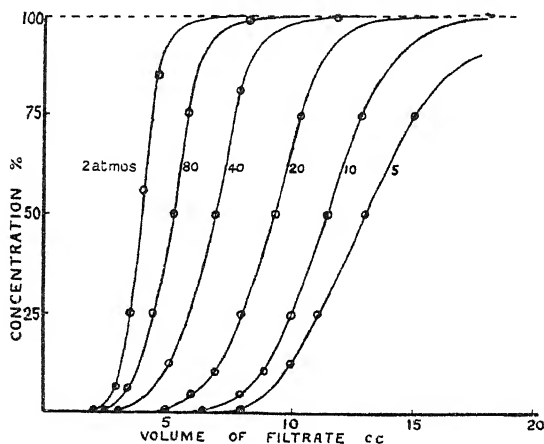


FIG. 2.—Filtration curves for 0.01 per cent. aqueous solution of night blue through membranes of A.P.D. 0.25 μ , thickness 0.15 mm. The figures 5 to 80 indicate the pressures in centimetres of mercury for their respective curves. The steepest curve was obtained with 2 atmospheres pressure. Filtration was in each case continuous, the pressure being steady and uninterrupted throughout.

particles are less firmly bound. The increased kinetic energy or momentum imparted to the filtering particles, as the pressure is augmented, results in their being less readily hindered by the frictional forces of cohesion within the capillary channels of the membrane.

With a polydisperse system, where a fraction of the dispersed material is withheld at the membrane surface purely by reason of the size of its particles in relation to the pore diameter, increased applied pressure will pack such retained particles into a compact surface layer, which may be less permeable than the membrane itself. Plainly, therefore, to augment the pressure in such a case will hinder rather than help the filtration of the more finely dis-

persed fraction. On the other hand, if very low pressures are used, the surface forces within the capillary channels can exert their maximum influence. In such circumstances there are doubtless optimum conditions, which can only be ascertained by experiment. In practice it is advisable to fractionate the system in stages, by stepping down the scale of membrane permeability gradually. Again, as McBain and Jenkins (1922) have pointed out for a membrane completely retaining the disperse phase, no liquid would theoretically be expected to filter until the osmotic pressure had been exceeded. The influence of pressure on the filtration of emulsions has been considered by Hatschek (1910).

The Effect of Capillary Active Substances.

The effect of capillary active substances in a disperse system, on the filterability of the disperse phase, is well illustrated by the action of soap in facilitating the passage of carbon black through filter paper. Ordinarily an aqueous carbon suspension is retained by filter paper, but the addition of soap solution enables the carbon particles to pass readily, and with collodion membranes a closely similar effect is found. Fig. 3 is a graph of data for experiments with

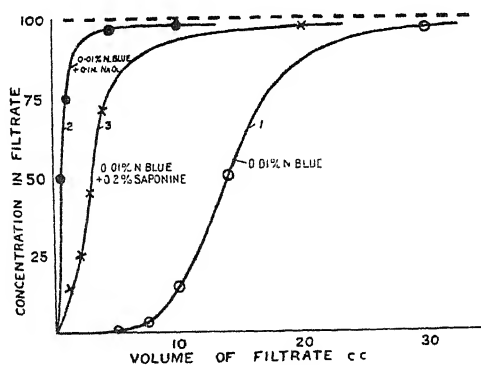


FIG. 3.—In each experiment the membrane was from 1.5 per cent. acetic acid collodion; the filtration pressure 10 cm. water.

0.01 per cent. night blue in water and a 1.5 per cent. acetic-collodion membrane. Curve 1 shows the course of filtration of the sol in pure water. Curve 2 is the corresponding result with the addition of 0.1 *n* NaOl. The adsorption zone has been practically eliminated, and the dye slips through the channels easily. The soap is more rapidly and completely adsorbed at the collodion/water interface than is the dye, while it also accumulates in the dye-particle/water

interface. The combined effect is analogous to lubrication; the frictional forces being minimized, the filtration is facilitated.

Similar effects are produced by other surface active materials, such as the saponins and bile salts, differing from those of the soaps only in degree, in accordance with specific surface activity. Clearly, therefore, the presence of a capillary active substance, even in quite low concentration, can have a profound influence upon filtration. The adsorption zone is reduced to small and possibly negligible proportions, so that the final process approximates much more closely to a true sieving action.

Brinkman and Szent Györgi (1923) found that collodion membranes, ordinarily impermeable to oxyhæmoglobin, were rendered permeable to this protein, if sodium oleate was first filtered through the membrane, or added to the hæmoglobin solution. It is clear that they were working with a membrane of such porosity that the failure of hæmoglobin to pass, in the absence of facilitation by sodium oleate, was due to adsorption. Had the pores of the membranes been smaller in diameter than the hæmoglobin particles, the capillary active substance would not have influenced the result. Fig. 4, Plate 17, illustrates this effect. It is a photograph of an ether-alcohol collodion membrane, through which a solution of oxyhæmoglobin had been filtered, without any of the protein appearing in the filtrate. Following this a dilute solution of sodium oleate was filtered through the same membrane. The oxyhæmoglobin became displaced from the pore surface and was washed through the effective capillaries, the filtrate becoming deeply coloured. The circular areas of membrane through which filtration was then occurring are clearly shown, the hæmoglobin having been almost completely removed from them.

Ward and Tang (1929) demonstrated an analogous effect with filter candles, in their studies with vaccinia and herpes viruses. When suspended in saline or Ringer solution the virus did not pass the filter at all, or only passed through with the greatest difficulty, but when "hormone broth" was used as the medium the virus passed through the filter readily. Similarly, Galloway and Elford (1931), in studying the filterability of the virus of foot-and-mouth disease through Gradocol membranes, found that with membranes of grade less than 100 $\mu\mu$ the filtration of the virus was facilitated by broth as compared with a phosphate-saline medium. The mechanism of the action of broth appears to be closely related to the property of this medium in stabilizing the dispersion of a lyophilic colloid. Soap may have an opposite influence, and certainly is harmful to bacterial and virus suspensions. With lyophobic sols, on the other

hand, broth usually shows a coagulating tendency, while soap generally exercises a stabilizing influence. The importance of dispersion stability on the filtration of suspensions has already been referred to. Further reference to the action of surface active substances will be made later, in its bearing upon the estimation of particle size from filtration data.

Filterability of Proteins.

Proteins, owing to their amphoteric character, can behave in aqueous solution as positive or negative colloids, and, although essentially lyophilic in nature may, under suitable conditions of hydrogen ion concentration and electrolyte content, exhibit the properties of lyophobic colloids in varying degrees. This diversity of behaviour is reflected in the filterabilities of proteins, which show some notable points of contrast when compared with those of lyophobic colloids. The viscosity of protein solutions results in slower rates of filtration, and the temperature coefficient of protein filtration is very appreciable. Hydrogen ion concentration is, perhaps, the most important factor in determining the inner equilibria of protein solutions, and a variation of filterability with p_H value is therefore to be expected. Fig. 5 shows the filtration curves for solutions of horse serum-albumin, adjusted to different p_H values by additions of hydrochloric acid and sodium hydroxide. Filtration occurs least readily at the isoelectric point. This fact suggests two things: (a) that the protein is in its highest state of aggregation when in the isoelectric condition, and (b) that it is most strongly adsorbed in this region. The former is in keeping with the maximum opalescence or light scattering exhibited by proteins in the neighbourhood of their isoelectric points (Krishnamurti, 1930), but, on the other hand, Svedberg (1930) from sedimentation analysis finds protein solutions to be uniformly monodisperse between characteristic limits of p_H . The dispersion, however, will also depend upon such factors as concentration and stability in relation to electrolytic content. As already pointed out potential instability in colloids is a factor of real importance in filtration, and, therefore, since proteins in general are most readily coagulated when in the isoelectric condition, this factor may contribute to the minimal filterability. The inference regarding the adsorption of protein is directly supported by the findings of several other workers, that isoelectric proteins are adsorbed by collodion membranes in maximum degree (Hitchcock, 1925; Risse, 1926; and Ettisch, Domontowitsch and v. Mutzenbecher, 1930). Further confirmation has been obtained from direct adsorption experiments of my own,

in which protein solutions were shaken with collodion particles prepared according to Loeb (1922) (see fig. 5). The preliminary results of a more extensive study of adsorption of proteins, from solutions of which the p_H values have been adjusted solely by use of hydrochloric acid and sodium hydroxide, have indicated the maximum adsorption in the isoelectric zone to be quite general for these conditions, and largely independent of the type of surface. This coincides with the maximum surface activity of isoelectric

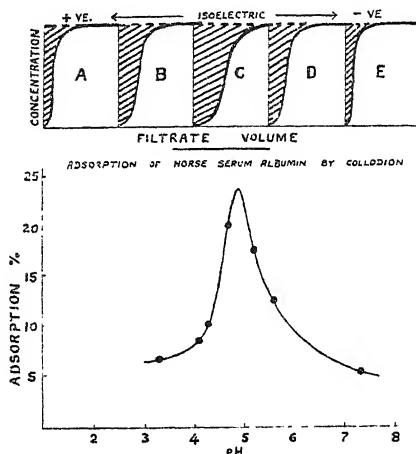


FIG. 5.—Curve of experimental results on the adsorption of 0.5 per cent. horse serum albumin by collodion particles. A pronounced maximum between p_H 4.5 and 5.0 corresponds with the isoelectric state of the protein. The filtration curves obtained with protein solutions of differing p_H values are shown diagrammatically. The adsorption zone is a maximum at the isoelectric point.

Adsorption $A < B < C > D > E$.

Rate of filtration $A > B > C < D < E$.

protein solutions, as shown by their minimum surface tension values. A further illustration of the sensitivity in behaviour of protein solutions is afforded by the action of oxyhaemoglobin; this, when buffered in M/15 phosphate, instead of adjusting the p_H with hydrochloric acid and sodium hydroxide, is adsorbed to a maximum extent on the acid side of the isoelectric point. The negatively charged collodion now preferentially adsorbs the positively charged protein. The effect is probably associated with some specific influence of the phosphate ion. Electrolytes are known to play an important rôle in determining the stabilities and degree of dispersion of colloids, and, as already mentioned, may, in conjunction with the hydrogen ion concentration, enable protein solutions to exhibit the properties of lyophobic colloids. The

evidence so far available suggests that the more lyophobic the protein colloid becomes, the less defined is the maximum adsorption in the isoelectric region. The adsorption/ p_H curve tends to take the \searrow form, greatest adsorption, and therefore impaired filtration, occurring on the acid side of the electrically neutral zone.

Filtration of Suspensions of Measured Particle Size, and the Relationship existing between the Pore Size and Particle Size at the Filtration End-Point.

The difficulties attending the application of ultrafiltration methods, to the determination of the size of particles in a suspension, will have become clear from the foregoing discussion. With a knowledge, however, of the manner in which the several factors operate it is possible to secure the conditions most favourable for filtration. It is necessary, when determining the end-point in membrane permeability for a given suspension, to establish the percentage-concentration/volume-of-filtrate curves for membranes of progressively lower porosities under standard conditions. It is possible then to ascertain the point at which surface forces of adsorption and cohesion begin to exert an abnormal influence upon the course of filtration. This indicates that the end-point is being approached, and is coincident with an observed rapid falling off in the rate of filtration during the experiment, owing to choking of the membrane. The curves of filtration from this point onwards must be studied with extreme care, selecting those conditions most favourable for filtration—optimum filtration pressure and minimum surface effects, as when a capillary active substance is present—until finally the value of the true filtration end-point is found. Plotting the maximum filtrate concentration against the respective pore sizes of membranes employed, a curve of the form in fig. 6 is obtained. The sharpness with which the end-point is defined depends upon the regularity of membrane structure and the uniformity of dispersion in the system being filtered. Experimental results with Gradocol membranes show that the first falling off in maximum filtrate concentration usually occurs at a pore size value varying from two to three times the absolute limiting value for non-filterability.

Experimental Results with Suspensions of known Particle Size.

B. prodigiosus.—This organism has been widely used in filtration work on account of its small and uniform size, and the ease with which it may be detected by cultural methods. Its smaller diameter, from microscopical

measurements, is found to range from 0.5 – $1.0\ \mu$. As already reported (Elford, 1931) *B. prodigiosus*, grown at room temperature for 48 hours on agar and then suspended in broth at p_H 7.6, is effectively retained by all membranes of pore size less than $0.75\ \mu$. The average pore size of the limiting membrane, therefore, gives a good indication of the size of the organism. It is important

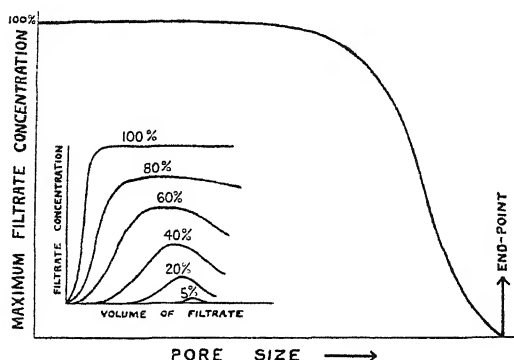


FIG. 6.—The small scale chart shows a typical series of filtration curves for membranes of progressively finer porosities from which the maximum filtrate concentrations may be obtained for the graph showing the end-point in membrane permeability.

to note, however, that particles as small as $0.5\ \mu$ are retained, so that the ratio particle-size/pore-size is 0.75 – 1.0 . The initial concentration of the bacterial suspension is found, further, to be an important factor in determining the end-point. It is very helpful, after having decided upon the most favourable filtration conditions, to construct a curve showing the minimum initial concentration of the disperse phase necessary to yield an active filtrate with a membrane of given porosity. Such a curve for *B. prodigiosus* is given in fig. 7, together with the corresponding curve for vaccinia virus (Elford and Andrewes, 1932). It is clearly indicated that above a certain porosity an active filtrate is obtained even with a very weak suspension, but that between this porosity and the true end-point an active filtrate is only assured when the initial concentration of the disperse phase exceeds a certain minimum value. Again, when the end-point is approached, the curve becomes very steep, until at the end-point the probability of a particle finding a pore sufficiently large for its passage vanishes, and the membrane holds back the particles completely.

Bovine Pleuro-Pneumonia.—A serum-broth culture of this organism, grown for 3 days at 37.5°C ., is found to consist of spherical bodies ranging in size from 0.2 – $0.5\ \mu$, together with a particulate phase which is below the resolution limits of the ordinary microscope. When such a culture, diluted in broth, is

filtered through membranes of progressively smaller pore size, the spheres can no longer be detected in filtrates from membranes of grade less than $0.35\ \mu$, although the particulate phase may be there and give rise to a typical culture in serum broth. Thus, confining attention to the spherical bodies, the limiting porosity is $0.35\ \mu$, which means that a membrane of this grade is able to retain particles as small as $0.2\text{--}0.25\ \mu$. The ratio particle size/pore size is therefore 0.6 to 0.75 . I am indebted to Mr. J. E. Barnard, F.R.S., for the microscopical examination of the filtrates on which the above determination is made, and for kindly confirming the size given for the organism.

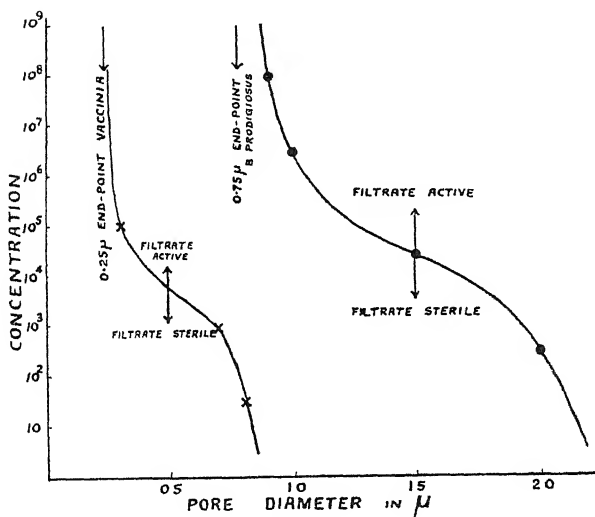


FIG. 7.

Gold Sols.—The results obtained with gold sols are summarized in Table II. The particle size in the sols was measured by the ultramicroscopical count method, which, it is well to remember, gives a value which is probably slightly higher than the true average. The filtration end-points have been determined at pressures up to three atmospheres, and with $0.001\ n$ sodium oleate in the sols to minimize surface effects. The relative particle sizes of the sols, indicated by filtration, are seen to follow the same sequence as the values given by the optical count method. The particle size/pore size ratio at the end-point is again definitely less than unity, having in the case of the most finely dispersed sol the value 0.5 .

Oxyhæmoglobin.—The end-point for oxyhæmoglobin from horse blood has been found to be $10\ \mu\mu$, all membranes below this limit completely retaining it, even in the presence of broth or sodium oleate. Svedberg (1930), by the

Table II.

Sol.	Method of preparation of sol from solution of gold chloride.	Appearance of sol.	Particle size by ultramicroscopic count method.	Filtration end-point in presence of 0.001N sodium oleate.	Ratio particle size/pore size
A	Reduction by formaldehyde in alkaline solution	Purple	$\mu\mu$ 50-60	$\mu\mu$ 80	0.625-0.75
B	Reduction by formaldehyde in neutral or very slightly alkaline solution	Ruby red	35	60	0.58
C	Reduction by sodium citrate...	Brilliant red	20	40	0.5

method of ultracentrifugal analysis, found the size of the haemoglobin particle in solution to be approximately $5 \mu\mu$, within the limits of p_H 6 to 8. It is interesting to note, also, that he found that the particle is not spherical, but possesses a dissymmetry ratio 1.25. Northrop and Anson (1929), by diffusion measurements, estimated the size of the oxyhaemoglobin particle to be $5.46 \mu\mu$. Thus the size of the retained particle is again about one-half the pore size of the limiting membrane.

Egg Albumin.—A 1 per cent. solution of recrystallized egg albumin, after filtration through a 0.6μ membrane to sterilize it, was diluted with its own volume of Hartley's broth at p_H 7.6, and filtered through graded membranes. The results are given in Table III.

Table III.

Membrane. A.P.D.	Thickness.	Pressure.	Maximum filtrate concentration.
$\mu\mu$	mm.	atmospheres	per cent.
9	0.081	3	75
7	0.055	3	10
5	0.050	3	Nil
4	0.050	4	Nil

The $4 \mu\mu$ membrane, although impermeable to egg albumin, permitted 75 per cent. of the broth constituents to pass. The end-point for egg albumin is therefore at about $6 \mu\mu$ porosity. Svedberg (1930), again by centrifugal analysis, found egg albumin solutions of p_H 4 to p_H 9 to be monodisperse, with molecular particles spherical in shape and $4.34 \mu\mu$ in diameter. The

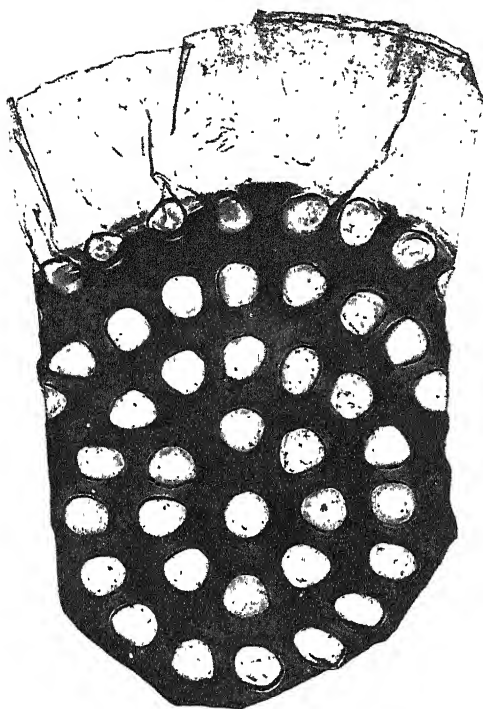


FIG. 4.

pore-size ratio for egg albumin is therefore 0.7, showing the disparity to be less than was found with hæmoglobin at 10 $\mu\mu$.

These experimental data, as to the relationship between the size of particles and the calculated pore diameters of membranes just effective in retaining them under the most favourable filtration conditions, are represented graphically in fig. 8. It is evident that for membranes above 1 μ grade, the average pore size gives a good indication of the size of the retained particle, although it must be remembered that the size of the largest pores is probably about twice the average value. As the pores become smaller, however, the membranes become more retentive than the assigned pore size would indicate, the particle/pore size ratio decreasing to 0.5 for a 10 $\mu\mu$ membrane. In this region the direction of the curve changes, and the disparity between particle and pore size becomes smaller again.

Before proceeding to consider the significance of these facts, the findings of other workers may be briefly reviewed. Krueger and Ritter (1930), using acetic acid collodion membranes, found a more or less constant difference, 50 to 75 $\mu\mu$, between the pore sizes indicated by the filtration of standardized

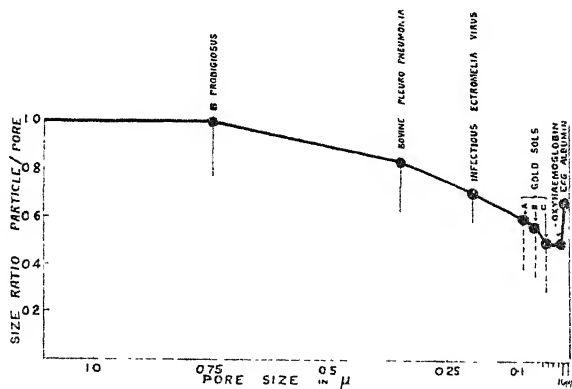


FIG. 8.—The points are for average values, the tails indicating the probable minimum range.

sols on the one hand, and the values calculated from Poiseuille's law on the other. Bechhold (1931) gave the relationship between the sizes of retained micro-organisms (bacteria and viruses) and the maximum pore size of filters (filter candles and membrane filters) to be 1/8 to 1/15, expressed as the ratio of the respective diameters. No special endeavour was made in these cases to ensure that optimum filtration conditions were being used, as regards both pressure and medium, and hence a greater disparity between particle size and

pore size is to be expected than that obtained in the present experiments. The fact that different types of filter were employed prevents any real comparison of results. It is significant, however, that the ultrafilters used were in all cases more effective in retaining microscopic and ultramicroscopic particles than would be anticipated from the calculated pore size, if the filter were regarded as a simple mechanical sieve.

Theoretical Expectations concerning the Particle/Pore Size Ratio for Limiting Filtration.

Let us consider an ideal system, in which the filter has uniform pores, which are circular in cross-section and run at right angles to the membrane surface, and in which the suspension is of uniform dispersion and the particles spherical and non-deformable. What is the size of the smallest particle, in relation to the calculated pore size, that such a filter can retain under optimum filtration conditions?

(a) *When adsorption occurs.*—The pore surface is pictured as having a certain adsorptive capacity which will be satisfied when a layer one particle thick

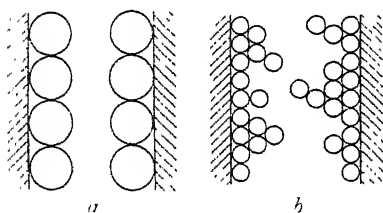


FIG. 9.

covers the surface, as in fig. 9A. This means that the pore becomes narrowed by an amount equivalent to twice the diameter of the adsorbed particle. Hence a particle can now be effectively retained by a pore of size originally three times its own diameter. Should the conditions be such that the adsorption is not confined

to mono-layer dimensions, but that, owing to residual cohesive forces, a layer several molecules thick may be formed, then the pore may retain particles much smaller than one-third its own diameter, fig. 9B. Yet it seems probable that the secondary layers, being less firmly attached than the primary layer, may yield or slip under the stress of a sufficient shearing force supplied by the filtration pressure. Thus, even with an ideal filter, if adsorption occurs, a pore may be expected to retain particles one-third its own diameter and most probably considerably less.

(b) *Where no adsorption of the disperse phase occurs.*—Although no adsorption takes place at the filter surface, resistance to filtration is offered by the mutually repelling force between the particles of the suspension and the pore walls, these carrying similar electric charges. This effect will be most pronounced

at low filtration pressures, and when the sizes of particles and pores concerned become comparable with the range of action of molecular forces, that is, in colloidal and molecularly dispersed systems. Increased filtration pressure assists the filtration of negatively charged metal sols through collodion membranes (also negatively charged in contact with aqueous solutions), probably because the increased kinetic energy imparted to the filtering stream of liquid enables the particle to shear past the resisting forces, which are immediately encountered at the entrance to the pore.

Conditions for no adsorption of the disperse phase obtain when a stabilizing capillary-active substance is present in the continuous medium. The surface forces are minimized, and adsorption of the disperse phase is eliminated, by the protective action of the adsorbed layer of surface active molecules around the particles. We may assume a layer one molecule thick of the protective substance lining the pore, and another coating the particle, giving four molecules in section through pore and particle. For limiting filtration conditions in such a system the following relationship will hold, assuming surface forces negligible :

$$p = d - 4s,$$

where

p = particle diameter,

d = pore diameter,

s = thickness of protective layer.

Hence, in systems where the capillary active molecule is small compared with the pore diameter, the relationship between pore size and particle size for limiting filtration should approximate to equality. As the systems become finer the thickness of the adsorbed protective layer becomes increasingly significant. To consider, for example, sodium oleate, Du Nouy (1926) has found the dimensions of the molecule of sodium oleate to be $12.3 \text{ \AA.} \times 7.56 \text{ \AA.} \times 6.64 \text{ \AA.}$ Since the orientation is with the major axis at right angles to the surface, $4s$ may be taken as $5 \mu\mu$.

Thus when

$$\begin{array}{lll} d = 100 \mu\mu & p = 95 \mu\mu & p/d = 0.95 \\ d = 10 \mu\mu & p = 5 \mu\mu & p/d = 0.5 \end{array}$$

Let us analyse in this way the filtration results obtained with oxyhæmoglobin. In the presence of sodium oleate this protein is retained by all membranes of average pore diameter less than $10 \mu\mu$. Neglecting the effects of surface forces and Brownian movement, in some measure compensated for by basing

the calculation upon the average and not the maximal pore diameter, the diameter of the oxyhæmoglobin particle by the foregoing analysis would be $5\text{ }\mu$. This figure is in agreement with the values, obtained by more quantitative physical methods, already quoted.

Even in the case of an ideally uniform system, therefore, a disparity between pore and particle sizes under the most favourable filtration conditions is anticipated. The effect both in magnitude and direction is essentially comparable with what has been found with membranes of average pore diameter down to $10\text{ }\mu$. The region of permeability below this limit has yet not been extensively investigated, attention having been focussed on the behaviour of suspensions with particles ranging from the microscopically visible down to colloidal dispersions—a zone within which the filterable viruses are found. In the case of egg albumin, however, the limiting pore size showed a closer approximation to the particle size than was observed with the somewhat larger particles of oxyhæmoglobin; whereas an increasing disparity might be theoretically expected, from the above argument, as the molecular state of dispersion is approached. The apparent conflict at this point, between experimental evidence and theoretical expectation, may be due to inaccuracies in the determination of pore diameter, which begin to be seriously significant as this degree of fineness in membrane porosity is reached. The sources of inaccuracy have not been dealt with above, and additional work would be necessary to assess their importance in measurements made with such fine membranes. A brief statement of the nature of the factors concerned may, however, be given here.

1. *The Volume of Effective Pores in Relation to Membrane Structure and Water Content.*—It is necessary in calculating the average pore diameter of a membrane to evaluate the total volume of the pores concerned. The assumption is made that the total pore volume is equal to the water content of the membrane, the proportion of “bound” in relation to “free” water being assumed negligible. In effect, the membrane is being regarded as having a structure consisting of parallel unidirectional pores at right angles to the membrane surface. Actually as we have already seen the existing structure is one in which the capillary channels intercommunicate. Now it can be shown that for membranes of high water content ($0.8\text{--}0.9\text{ c.c./cm.}^3$) that the pores must of necessity be so closely packed that the volume corresponding to the effective pores perpendicular to the membrane surface accounts almost completely for the total water content. The volume not common to the effective pores, owing to pores running in other directions, is such a very small

fraction of the total volume, that, for practical purposes of membrane calibration, the assumption that the total pore volume equals the water content is quite justifiable. Experimentally we find that membranes have high water content values ranging from 0.92 to 0.80 c.c./cm.³ over a wide range of pore diameter, 2 μ down to the region of 10 $\mu\mu$. In the latter region, however, the water content begins to fall off precipitously, the proportion of solid material in the membrane showing a correspondingly rapid increase, as the membranes become still less permeable. The error involved in estimations of pore diameter based upon the assumption that the total volume of the pores is equivalent to the water content will no longer remain negligible (less than 5 per cent.), but will result in the estimated pore diameter values of membranes, in this region of fine porosity, being too low. A full analysis of this effect must await the accumulation of more data with these very fine membranes.

2. *Electrokinetic Effects*.—The flow of water through a fine capillary is opposed by a “back endosmotic velocity” due to the streaming potential produced by the forward motion of the liquid. Experiments are being made to try to obtain data which will enable the magnitude of this effect in the case of membranes to be estimated. For glass capillaries it becomes appreciable when the diameters are less than 1 μ , but the significance of this effect may be quite different for collodion membranes, whose structure is not comparable to a system of insulated parallel capillaries, since the channels are being frequently short-circuited by the intersecting channels in other directions. Nevertheless, it is clear that the action of such a retarding influence upon the rate of flow would result in the estimated pore size being lower than the true value.

3. *Applicability of Poiseuille's Law for Ultramicroscopic Capillaries*.—The assumption that the influence of the immobilized molecules at the pore surface is negligible may be justified when the capillary dimensions are large compared with the immobilized layer of molecules. Eventually, however, their influence must be felt, possibly either in the form of an electro-viscous resistance to flow, or solely through spacial relationships. The space occupied by a layer of water molecules may narrow the pore to the extent of nearly 10 per cent. in a capillary of 10 $\mu\mu$ diameter, and, should normal viscosity not be maintained to within molecular distance of the pore wall, the added electro-viscous resistance to flow will make the apparent pore size even smaller.

It is significant that each of the three factors mentioned operate to make the estimated average pore diameter smaller than the true pore diameter, and their combined effect may be expected to become serious in membranes of porosities below 10 $\mu\mu$. The apparent closer approximation of pore diameter to the

diameter of the filtered particle, observed in the case of egg albumin, may be ascribed, at least in part, to such an underestimation of the average pore diameter in this region.

Estimations of Particle Sizes from Filtration End-points.

Since the several determining factors in filtration cannot be independently assessed with accuracy, I have adopted the practical procedure of expressing the probable limits for the size of the smallest particle just retained by a given membrane, as obtained *under the best available conditions for filtration*. The relationship between particle and pore size, given in Table IV, is based upon the experimental evidence shown in the curve of fig. 8, in conjunction with the theoretical expectations.

Table IV.

Membrane average pore diameter.	Size of retained particle.
$\mu\mu$	
10-100	(0.33-0.5) d
100-500	(0.5-0.75) d
500-1000	(0.75-1.0) d

d = average pore diameter of limiting membrane for optimum filtration conditions.

It will be observed that the factor determining the particle size, from the observed end-point in membrane grade, varies with the region of porosity. The factor is smallest for the zone 10 to 100 $\mu\mu$, corresponding with maximum colloidalilty in dispersion, but increases with the permeability toward unity. This is justified both by experimental evidence, and by the known manner in which such factors as kinetic or Brownian movement, specific surface forces, and the general stability of suspensions vary with particle size. A calibration curve of reference would be ideal but, in view of the practical difficulties of ensuring strictly uniform filtration conditions, the only justifiable procedure is to give the probable limiting size values, thus allowing tolerance for the inherent difficulties of the method. Some of the estimations made on this basis are given in Table V, which also shows the values given by other methods. The agreement is good, but estimations of particle size from filtration data should always be checked by other physical methods, when these are available. Opportunity for testing the reliability of the filtration method is being furnished by studies of the sizes of virus particles. The first of these to be completed was that of the virus of infectious ectromelia (Barnard and Elford, 1931). The size of the virus from filtration data was found to be 0.10-0.15 μ , while

Table V.

Substance.	End-point.	Probable size by filtration.	Size by other methods.
<i>B. prodigiosus</i>	μ 0.75	μ 0.5-0.75	0.5-1.0 μ microscopically.
Bovine <i>P. pneumonia</i> — Spheres Particles	0.35 0.20	0.175-0.25 0.1-0.15	0.2-0.5 μ microscopically (Barnard). >0.2 μ
Vaccinia virus . .	0.25	0.125-0.175	0.21-0.23 μ centrifugal analysis (Bechhold and Schlesinger, 1931). 0.17-0.18 μ (max.) U.V. light photography (Barnard).
Infectious ectromelia virus .	0.2	0.1-0.15	0.13-0.14 μ ultra-violet light photography (Barnard).
Herpes virus	0.2	0.1-0.15	
Gold sol (purple) .	$\mu\mu$ 80	$\mu\mu$ 30-40	50-60 $\mu\mu$ ultramicroscopical count.
Phage C 36	65	20-30	Bechhold and Villa (1926), <i>B. coli</i> phage 35 $\mu\mu$, optical method. Hetler and Bronfenbrenner (1931), <i>B. coli</i> phage 22.8-1.2 $\mu\mu$ by diffusion.
Gold sol (red)	40	15-20	20 $\mu\mu$ ultramicroscopical count.
Foot and mouth disease virus .	25	8-12	
Oxyhæmoglobin	10	3-5	5 $\mu\mu$ ultracentrifugal analysis (Svedberg). 5.4 $\mu\mu$ diffusion (Northrop and Anson).
Egg albumin	6	—	4.34 $\mu\mu$ ultracentrifugal analysis (Svedberg).

the subsequent measurements of real photographic images of the virus bodies enabled Mr. Barnard to calculate their size as 0.13-0.14 μ . The more recent results with vaccinia virus have also shown that the evidence from the two methods is in good agreement. Elford and Andrewes (1932) found the probable size of the elementary virus units to be 0.125-0.175 μ by filtration, while Mr. Barnard finds 0.17-0.18 μ to be the maximum size by ultraviolet light photography.

Summary.

(1) The principles involved in filtration through ultrafilter membranes have been discussed from an essentially practical standpoint. The several factors concerned in the general process of filtration have been investigated using typical disperse systems—colloidal dyes, metal sols, protein solutions and suspensions of bacteria and viruses.

(2) The relationship between the size of retained particles and the estimated pore sizes of membranes is also discussed. The fact that, for colloiddally dispersed systems, the pore size of the limiting membrane is definitely greater than the size of the retained particle, even under optimum filtration conditions, is shown to be in accordance with theoretical expectations.

(3) The particle sizes of suspensions, estimated from filtration experiments with carefully graded collodion membranes, are found to agree well with the values obtained by other methods. The method of analysis adopted has proved of great value in virus studies.

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*On the Evidence Against the Chemical Induction of Melanism in
Lepidoptera.*

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1. *Introductory.*

McKenny Hughes (1932) has reported an experiment, carried out at Merton, in which the moth *Selenia bilunaria* Esper was fed on leaves treated with lead nitrate and manganous sulphate. In the generations following these treatments no instance of a moth showing the melanic recessive mutation, reported by Harrison and Garrett (1926), was recorded.

In the discussion contributed by Haldane (McKenny Hughes (1932), p. 400) it is argued that the results are significantly in conflict with the findings of Harrison and Garrett in that these authors recorded 6 melanics out of 142 moths of the generations following treatment, while McKenny Hughes found no melanics among 910 moths. This difference, as Haldane claims, would be highly significant, if the several individuals counted had an independent chance of being melanics. In both lots, however, the moths were in reality closely related, and the chances cannot on any theory be considered independent. The other calculations in Haldane's discussion are open to the same criticism.

The value of a negative finding, unlike that of a positive result, such as that reported by Harrison and Garrett, rests exclusively on the extent of the evidence. The different broods reported by McKenny Hughes are not only of different sizes, but of several different kinds, having very different weight as negative evidence. Thus, to take the lead series only, in family C there are 21 broods, comprising 132 moths, all in the first generation following treatment with lead. In family D there are (a) 12 broods comprising 220 moths in the first generation following treatment, (b) two broods of 49 moths after two generations of treatment, and (c) one brood of 25 moths the parents of which had been off lead for one generation. Finally, in family G there are (a) 14 broods comprising 209 moths in the first generation following treatment, (b) 8 broods comprising 138 moths after two generations of treatment, (c) 8 broods comprising 43 moths off lead for one generation, (d) 12 broods comprising 83 moths after three generations of treatment, and (e) 2 broods comprising 7 moths off lead for two generations.

Table I shows the combined totals for the three families.

Table I.—Number of broods and moths bred.

	One generation treatment.	Two generations treatment.	Three generations treatment.	Off lead one generation.	Off lead two generations.	Total.
Broods	47	10	12	9	2	80
Moths	561	187	83	68	7	906

The small discrepancies from Haldane's values are presumably to be ascribed to errors of transcription. It will, however, make no appreciable difference whether the error is in the tables printed with McKenny Hughes' paper, or in the totals given by Haldane.

The value of the material as negative evidence may be measured by the rates of mutation which, in the light of the observations, can be shown to be highly improbable. An infinitude of observations would be required to show that lead treatment had actually zero effect on the mutation rate. Any body of data showing no mutations may be accepted as proving the non-existence of mutation rates above a certain critical level; the more extensive the observations, and the more relevant they are to the point at issue, the smaller will this critical level be made. In particular we may ask, for the body of material presented, for what mutation rate the probability of observing no melanic moths will be reduced to 0.05 or 0.01. The experiment might then be interpreted as proving, with degrees of certainty measured by these two levels of significance, that the mutation rate did in fact not exceed the corresponding value found. These critical mutation rates will be evaluated in the following sections.

2. *Definition of Mutation Rate and Form of Calculation.*

The probability of the absence of visible mutants in a given series of affiliated progenies will be determined not only by the mutation rate, but by the stage at which the mutations are supposed to occur. By hypothesis A we shall denote the view that a mutation rate μ per generation implies that a non-mutant individual will after treatment produce a fraction μ of mutant gametes, and a fraction $(1 - \mu)$ of non-mutant gametes. A heterozygous individual, on the other hand, will produce $\frac{1}{2}(1 + \mu)$ mutant gametes, and $\frac{1}{2}(1 - \mu)$ non-mutant gametes. There will, therefore, on this hypothesis, be only three types of mating to be considered, as shown in Table II.

Table II.—Frequency of the three kinds of offspring from the different types of mating.

Mating.	Offspring.		
	MM.	Mm.	mm.
MM × MM	$(1 - \mu)^2$	$\frac{2\mu(1 - \mu)}{1}$	$\frac{\mu^2}{1}$
MM × Mm	$\frac{1}{2}(1 - \mu)^2$	$\frac{1}{2}(1 - \mu)(1 + 2\mu)$	$\frac{1}{2}\mu(1 + \mu)$
Mm × Mm	$\frac{1}{4}(1 - \mu)^2$	$\frac{1}{2}(1 - \mu^2)$	$\frac{3}{4}(1 + \mu^2)$

Thus if μ is 3 per cent., the proportionate frequencies of melanic moths in the three types of mating will be 0.0009, 0.01545, 0.265225. and if the progeny yields s moths, the probabilities that all shall be non-melanic will be $(0.9991)^s$, $(0.98455)^s$ and $(0.734775)^s$. Columns (c), (d) and (e) of Table III give, in reverse order, these probabilities for the sixth generation (the third after treatment), using $\mu = 3$ per cent. for the offspring of treated moths, and $\mu = 0$ for the offspring of the untreated. It will be observed that families over ten have only a small probability of being the offspring of two heterozygotes, but that even the large family of 33 might well have been the offspring of a heterozygote and a homozygote.

The probabilities of the three possible matings producing the sixth generation may be used to calculate the probabilities of these same alternatives in the fifth generation. Thus from a mating of homozygotes the probability that both the offspring chosen for mating shall be homozygotes will be $(1 - \mu)^4$, the probability that one shall be a homozygote and one a heterozygote will be $4\mu(1 - \mu)^3$, and the probability that both shall be heterozygotes will be $4\mu^2(1 - \mu)^2$. If, therefore, the mating, which produced the fifth generation progeny, was one between non-mutant moths, the probability that a pair of its members mated *inter se* will produce s offspring non-mutant in appearance, will be

$$4\mu^2(1 - \mu)^2 \left\{1 - \frac{1}{4}(1 + \mu)^2\right\}^s + 4\mu(1 - \mu)^3 \left\{1 - \frac{1}{2}\mu(1 + \mu)\right\}^s \\ + (1 - \mu)^4 \{1 - \mu^2\}^s,$$

or, with a 3 per cent. mutation rate, the sum of the values in columns (c), (d) and (e) of Table III, multiplied in order as they stand by 0.003387, 0.109521, 0.885293, respectively. The values in column (h) in Table III have been obtained by using these factors.

Table III.—Calculation of probabilities—first stage.

Sixth generation brood (a)	Moths. (b)	Fifth generation mating.			Fourth generation mating.			Brood of parents. (i)
		Mm × Mm. (c)	MM × Mm. (d)	MM × MM. (e)	Mm × Mm. (f)	MM × Mm. (g)	MM × MM. (h)	
GL18/30J	1	0.73478	0.98455	0.99910	0.47003	0.89157	0.99481	} GL17/30S
GL17/30J	1	0.73478	0.98455	0.99910	0.47003	0.89157	0.99481	
GL14/30J	7	0.11563	0.89674	0.99371	0.29458	0.68426	0.97833	
GL13/30J	3	0.39670	0.95436	0.99730	0.37847	0.78721	0.98877	} GL12/30S
GL16/30J	2	0.53989	0.96934	0.99820	0.41777	0.83250	0.99169	} GL11/30S
GL12/30J	5	0.21418	0.92510	0.99551	0.32594	0.72442	0.98336	
GL15/30J	33	0.00004	0.59820	0.97072	0.19431	0.21484	0.92489	} GL7/30S
GL8/30J	10	0.04587	0.85581	0.99104	0.26741	0.64543	0.97125	
GL11/30J	12	0.02476	0.82957	0.98925	0.25587	0.62676	0.96672	} GL6/30S
GL9/30J	7	0.11563	0.89674	0.99371	0.29458	0.68426	0.97833	
GL5/30J	1	0.73478	0.98455	0.99910	0.47003	0.89157	0.99481	} GL2/30S
GL4/30J	1	0.73478	0.98455	0.99910	0.47003	0.89157	0.99481	
GL10/30J	5	0.23730	1	1	0.37182	0.80032	1	} GL4/30S
GL3/30J	2	0.56250	1	1	0.45312	0.89062	1	

Similarly column (g), representing the supposition that the fifth generation brood was derived from the cross of a homozygous non-mutant with a heterozygote, is obtained by multiplying the same quantities by the factors

$$\frac{1}{4}(1 - \mu)^2(1 + 2\mu)^2, \quad \frac{1}{2}(1 - \mu)^3(1 + 2\mu), \quad \frac{1}{4}(1 - \mu)^4,$$

or numerically by 0.264299, 0.483717, 0.221323 for a 3 per cent. mutation rate, or by 0.25, 0.50, 0.25 for zero mutation rate.

Finally, the factors for the supposition that the fifth generation brood was derived from the mating of two heterozygotes are

$$\frac{1}{4}(1 - \mu^2)^2, \quad \frac{1}{4}(1 - \mu^2)(1 - \mu)^2, \quad \frac{1}{16}(1 - \mu)^4,$$

or, numerically, 0.249550, 0.235013, 0.055331 and 0.25, 0.25, 0.0625 for $\mu = 0.03$, and zero respectively. These factors are used to obtain the values in column (f).

We are now in a position, for any progeny of the fifth generation, to express the probability of each of the three possible types of mating from which it might have been derived, taking into account not only the composition of the fifth generation, but also of that of the broods derived from it. Thus brood

GL17/30S consists of 15 moths of which three pairs have been used in the production of the sixth generation broods GL18/30J, GL17/30J and GL14/30J. Apart from the sixth generation the probability that this fifth generation brood had been derived from a mating of two heterozygotes would have been $(0.734775)^{15}$; taking the sixth generation into account it is now seen to be $(0.734775)^9$, $(0.47003)^2$, $(0.29458)^1$, or 0.00406 as set down in Table IV. The index of the first power is 9 and not 15, since the chance that the 6 moths used as parents should be non-mutants has already been taken into account in the three corresponding factors. Columns (c), (d) and (e) of Table IV, derived thus from columns (f), (g) and (h) of Table III, show that many of the fifth generation progenies were sufficiently large to exclude, as improbable, the idea that they were from the mating of two heterozygous mutants; while for only one of the progenies, GL7/30S, a large brood from which two satisfactory broods had been derived, is there shown a low probability of its having one mutant parent.

Table IV.—Calculation of probabilities—second stage.

Fifth generation brood. (a)	Moths not mated. (b)	Fourth generation mating.			Third generation mating. (h)	Brood of parents. (i)
		(c)	(d)	(e)		
GL17/30S	15-6	0.00406	0.47280	0.96038	0.90200	GL16/29J
GL11/30S	30-4	0.00004	0.40231	0.95262	0.88741	GL15/29J
GL16/30S	4	0.29148	0.93962	0.99640	0.98600	GL14/29J
GL12/30S	19-2	0.00201	0.60414	0.97375	0.92823	} GL11/29J
GL7/30S	29-4	0.00002	0.09395	0.87830	0.78784	
GL6/30S	18-4	0.00101	0.34487	0.93391	0.86456	
GL5/30S	1	0.73478	0.98455	0.99910	0.99481	GL6/29J
GL2/30S	22-4	0.00086	0.60061	0.97373	0.92782	GL3/29J
DL7/30S	14	0.01337	0.80414	0.98746	0.96231	DL11/29J
DL5/30S	35	0.00002	0.64665	0.96894	0.92862	DL17/29J
GL15/30S	9	0.07508	1	1	0.99507	} GL7/29J
GL10/30S	6	0.17798	1	1	0.99542	
GL9/30S	2	0.56250	1	1	0.99672	
GL8/30S	1	0.75000	1	1	0.99735	
GL14/30S	12	0.03168	1	1	0.99492	} GL10/29J
GL13/30S	6	0.17798	1	1	0.99542	
GL4/30S	6-4	0.09477	0.72081	1	0.96456	} GL4/29J
GL3/30S	1	0.75000	1	1	0.99735	
DL1/30S	25	0.00075	1	1	0.99482	DL7/29J

For an experiment extending over many generations the cycle of operations set out above may be repeated indefinitely. Since in the experiment under discussion, the third generation was the first to be treated, the only hypothesis to be considered as to the origin of the fourth generation is that its parents were in each case both homozygous non-mutants. Consequently, columns (*f*) and (*g*) will not be needed in Table IV, and only column (*h*) will have to be calculated; the formula for treated moths is at this stage used in every case. The 19 pairs of the fourth generation, which had progeny, have accounted for 38 moths, leaving 523 others each with a probability 0.9991 of being a mutant. The product of the 19 values shown in column (*h*) of Table IV, multiplied by 0.9991 to the power of 523, will therefore give the probability of the observed negative result, on the hypothesis of a 3 per cent. mutation rate specified by hypothesis A.

The product of the 19 values comes to 0.43209, and the power of 0.9991 to 0.62446. The final probability of the whole series of observations is therefore 0.26982; hence the experiment is far from excluding the possibility of a mutation rate of 3 per cent.

On repeating the calculations with a 10 per cent. mutation rate, the product of the contributions of the 19 families of the fifth generation is 0.0013216, while 0.99 to the power of 523 contributes a factor 0.0052145. The probability of such a negative result as that observed, if there had been a mutation rate, as defined, of 10 per cent., is only 6.8915 millionths. The observations thus effectively exclude this possibility.

The logarithm of the probability evidently increases, in the range considered, more rapidly than the first power, though not quite so rapidly as the second power of the mutation rate. Interpolating logarithmically, the 1 per cent. point is found to be at a mutation rate of about 6.0 per cent., and the 5 per cent. point at about 4.7 per cent.

Since the logarithm of the probability increases proportionately with the bulk of the data, supposing these to be of the same kind, it may also be inferred that to exclude mutation rates exceeding 1 per cent. per generation would have required about 17 times as many observations as those reported if we were satisfied with the 5 per cent. level of probability, or about 26 times as many to reduce the probability to 1 per cent.

3. *Alternative View of the Action of the Mutations.*

The data of Harrison and Garrett are not in accordance with the possibility that some 5 per cent. of the gametes of moths treated as larvæ carry the mutant

gene, for then only about 1 in 400 of each brood of the second generation could show the mutation. On the contrary, Harrison and Garrett reported no broods without melanics, and two broods showing 3 melanics and 55 normals. Data of this kind suggest that the mutation, if not already present in the stock, occurred at an earlier stage than has been supposed, so that when a mutation is induced a large proportion of the gametes are affected. We may therefore consider the alternative supposition (B), or, rather, the other extreme of the range of possibilities; namely, that a single mutation affects the whole germ tract, so that a treated non-mutant has a probability $(1 - \mu)^2$ of propagating as a non-mutant, a probability $2\mu(1 - \mu)$ of propagating as a heterozygote, and a probability μ^2 of propagating as a homozygous mutant. This extreme supposition is not that best suited to Harrison and Garrett's data, for it would require those broods in the second generation which contain melanics to show 25 per cent. melanics; actually they show only about 5 per cent., which would be near to expectation if a single mutation affected about half the germ tract of the animal in which the mutation took place. We shall not, however, consider this special possibility, since its characteristics will doubtless be brought out by the more extreme and definite hypothesis to be considered.

The practical computation is, in the case of hypothesis B, a little more complicated than in hypothesis A since there are 5 instead of 3 types of mating to be considered. The two additional types arise when individuals normal in appearance are transformed as progenitors into homozygous mutants. Two types of mating give a proportion of mutant offspring; namely, $Mm \times Mm$ giving 25 per cent. melanics, and $Mm \times mm$ giving 50 per cent. The probabilities of a mating yielding s normal individuals being of these kinds are therefore given by the values $(\frac{3}{4})^s$ and $(\frac{1}{2})^s$ respectively.

A normal individual from either the mating $MM \times mm$ or $Mm \times mm$ is bound to be heterozygous before mutation, and after treatment will be germinally heterozygous in $(1 - \mu)$ cases, and homozygous in μ cases. A pair of such individuals mated will therefore give a mating of type $Mm \times Mm$ in $(1 - \mu)^2$, of type $Mm \times mm$ in $2\mu(1 - \mu)$ and of type $mm \times mm$ in μ^2 cases. Hence the contribution of any progeny of s to the probability that the parent progeny was of either the types $MM \times mm$ or $Mm \times mm$ will be

$$\mu^2 (0)^s + 2\mu(1 - \mu) (\frac{1}{2})^s + (1 - \mu)^2 (\frac{3}{4})^s,$$

which we may write in a more general notation as

$$2\mu(1 - \mu) p_{011} + (1 - \mu)^2 p_{121},$$

where p stands for the probability, as judged by its observed composition, and that of its descendants, that a progeny is of the theoretical composition indicated by the suffix.

Similarly if the parent progeny is of type $Mm \times Mm$, the probability that a normal offspring will propagate as a homozygous normal is $\frac{1}{3}(1 - \mu)^2$, as a heterozygote $\frac{2}{3}(1 - \mu^2)$, and as a homozygous mutant $\frac{1}{3}\mu(2 + \mu)$. The contribution of the offspring of a pair of such normal offspring to the probability that the parental mating is of this type will therefore be

$$\frac{1}{3}\mu(2 + \mu)(1 - \mu^2)p_{011} + \frac{4}{9}(1 - \mu^2)^2p_{121} + \frac{2}{3}\mu(2 + \mu)(1 - \mu)^2p_{010} \\ + \frac{1}{9}(1 - \mu^2)(1 - \mu)^2p_{110} + \frac{1}{9}(1 - \mu)^4p_{100}.$$

In like manner the contribution to the probability that the parental mating is of type $MM \times Mm$ is

$$\frac{1}{2}\mu(1 + 2\mu)(1 - \mu^2)p_{011} + \frac{1}{4}(1 - \mu)^2(1 + 2\mu)^2p_{121} + \frac{1}{2}\mu(1 - \mu)(1 - \mu^2)p_{010} \\ + \frac{1}{2}(1 + 2\mu)(1 - \mu)^3p_{110} + \frac{1}{4}(1 - \mu)^4p_{100},$$

and the contribution to the probability that it is $MM \times MM$ is

$$4\mu^3(1 - \mu)p_{011} + 4\mu^2(1 - \mu)^2p_{121} + 2\mu^2(1 - \mu)^2p_{010} \\ + 4\mu(1 - \mu)^3p_{110} + (1 - \mu)^4p_{100};$$

so that starting from the terminal progenies we may calculate, as before, the probability of the series of non-melanic progeny observed for any chosen mutation rate.

Using a mutation rate of 10 per cent. the probability of the series of normal families observed by McKenny Hughes is found to be 0.0097551, so that 10 per cent. per generation is just over the 1 per cent. value for hypothesis B. One obvious reason for the lower sensitivity of the experiment to hypothesis B, compared with hypothesis A, is that in the first generation following treatment the chance of showing no mutant for a brood of 1 is 0.99 on both hypotheses. On hypothesis A, however, any further members of the brood have an equal and independent chance, so that the probability of s non-mutants is $(0.99)^s$; while on hypothesis B the probability of a non-mutant brood, however large, cannot fall below 0.9639, for this is the probability that one or other of the parents is a non-mutant. The 21 broods of Series C, for example, comprise 132 normal moths, but the probability on hypothesis B of this series of observations is 0.57372, equal to that of about 55 individual moths on hypothesis A, or to 55 broods of one on hypothesis B. Many other examples show emphatically how nearly impossible it is by judgment alone, and without explicit calculations, to gauge the value of negative evidence of this kind.

4. Discussion.

The observations reported by McKenny Hughes are sufficiently extensive to exclude as improbable mutation rates exceeding 6.0 per cent. per generation induced by lead treatment, on the hypothesis that mutations are induced independently in the gametes; for the series of normal families observed would have a probability of occurrence of less than 1 per cent. if the mutation rate had exceeded this value. Less decisively, that is, with a 5 per cent. level of significance, are mutation rates exceeding 4.7 per cent. excluded.

Using the same sort of observations, the amplitude of the material would have to be increased 17-fold in order to exclude mutation rates over 1 per cent. on the lower standard, and about 26-fold to exclude them on the higher standard of significance.

On the alternative view that the mutations affect not individual gametes independently, but the whole germ tract of the individual affected, the observations are still less conclusive, for the probability of the series of normal families observed is only just under 1 per cent. with a mutation rate as high as 10 per cent.

Harrison and Garrett, after a single generation of treatment, mated four moths, all of which acted as partial heterozygotes. Apart from exceptional good fortune, this suggests an enormous mutation rate; for, with a mutation rate of 8 per cent., only about 30 per cent. of the treated moths should act as semi-heterozygotes. With *Tephrosia bistortata*, on the other hand, only a single brood after four generations of treatment yielded a melanic, a result suggestive of a much lower, though still absolutely large, mutation rate.

It is not the writer's purpose to attempt to justify the very remarkable claim put forward by Harrison and Garrett, and it appears by no means impossible that the mutants observed were in reality segregates from a mutation pre-existing in the stock. If it is true that the melanics are less viable than normal moths, the paucity (5 per cent. against 25 per cent. expected) of melanics in the broods in which they first appeared would be explained. The system of experimentation adopted at Merton is, however, quite insufficient to show that chemical agencies do not induce mutations with even more than the high mutation rate of 1 per cent.

To view the matter in perspective we may note that of known physical agencies, the most effective in inducing mutations, namely, irradiation with X-rays, seldom causes a rate of more than one in several thousands at a particular locus. Thus Timoféeff-Ressovsky (1930), whose work I cite as pre-eminent in its thoroughness and extent, reports 15 back mutations in *Drosophila*

melanogaster, out of 213,567 irradiated chromosomes, or a little less on the average than 1 in 14,000. Even to establish the absence of mutation rates exceeding 1 per cent., though sufficient to require a reinterpretation of Harrison and Garrett's observations, would only show that the chemical agency (lead) is not more than 140 times as effective as X-rays are ordinarily found to be.

Had it been possible to test for mutations not by inbreeding only, but by back-crosses to the melanic form, little difficulty should have been encountered, with material not considerably greater than that used by McKenny Hughes, in excluding mutation rates over 1 per cent. For, without inbreeding, but using crosses between different treated families, and in the absence of disease, there can be little doubt that satisfactorily large broods, at least exceeding 10 moths, would have been readily obtained after three generations of treatment. A hundred such broods formed by crossing treated moths with melanics would test 200 chromosomes. With a mutation rate of 1 per cent. for three generations, about 3 per cent. of these should contain mutant genes, on either view of the incidence of mutation, and the absence of all melanics, when six affected families were expected, would be good evidence against a mutation rate of 1 per cent.

The method of attempting to reveal possible mutations by inbreeding, not only introduces difficulties by impairing the stock, but is exceedingly inefficient compared to back-crossing. Since all scientific experiments are limited by the amount of money and labour which can be expended upon them, it is highly desirable that any experiment should be designed so as to use the available resources to the best advantage.

Summary.

A method is given of assessing by calculation the value of evidence of the non-occurrence of recessive mutations under experimental conditions. It appears that the evidence, against the induction of melanic mutations in moths by feeding with lead, is insufficient to disprove the existence of mutation rates up to 5 per cent. or 8 per cent. according to the stage at which mutation is postulated.

Mutation rates of this magnitude would be far greater than those which can be certainly induced by any other agency.

The use of back-crosses instead of inbreeding would increase the value of experimental data of this kind by approximately thirty-fold.

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Chondrogenesis in Cultures of Endosteum.

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[PLATES 18–20.]

Introduction.

In earlier work on the histogenesis of the fowl skeleton (Strangeways and Fell, 1926 ; Fell, 1928), it was shown that nodules of small-celled, non-ossifying cartilage developed very readily in cultures of limb-bud mesoderm and that in very rare cases (Fell, 1928) the chondroblasts hypertrophied and ossification occurred. More recently (Fell, 1932) similar nodules of small-celled cartilage were found to develop in cultures of early (6-day) embryonic periosteum. Studitsky (1932), using the method of chorio-allantoic grafting, has obtained chondrogenesis in grafts of later (15–19 day) embryonic periosteum, but he does not specify what type of cartilage developed.

In previous experiments (Fell, 1932) cultures *in vitro* of endosteum from the tibiae of hatched chicks always produced bone or osteoid tissue but chondrogenesis was not observed. In the course of later investigations, however, some striking examples of the formation of hypertrophic cartilage in endosteal cultures were obtained, and the present communication describes the development of such cartilage, and its subsequent direct transformation into osteoid tissue or bone.

Material and Methods.

The cultures were derived from the middle region of the tibiae of six post-embryonic chicks, one aged 13 days, two 6 days, one 7 days, and one 4 days, whilst a single culture, the first chondrogenic culture obtained, was derived from the tibia of a 3-day bird. As previously described (Fell, 1925), endochondral ossification in the long-bones of the fowl is confined to the extreme ends of the diaphysis, fig. 1, *a*, Plate 18, and in a young chick the cartilage occupying the greater part of the shaft is completely excavated within the tube of periosteal bone and replaced by marrow. In the present experiments only this completely excavated part of the bone was used so that no cartilage

was present in the original explants. After the cartilaginous ends had been discarded, the periosteum was stripped from the outer surface of the bone which was then split in half and the marrow scraped away. The two halves were again split longitudinally and each of the four strips of bone thus produced was cut transversely into a series of small rectangles, the longest sides of which were at right angles to the long axis of the shaft. In order to demonstrate the complete absence of cartilage from the region of the shaft from which the explants were taken, in the last experiment of the series, fig. 1, one entire tibia, and the rejected ends of the corresponding tibia from the opposite side, were fixed and sectioned at a thickness of $25\ \mu$ as controls, whilst the piece of shaft from which the ends had been cut was used for the preparation of cultures. The sections of the control material were stained with thionin, which rendered the cartilaginous areas strikingly conspicuous owing to the dense metachromatic colouration of the cartilage matrix. As may be seen from fig. 1, *a*, Plate 18, the middle region of the shaft in the complete tibia contains marrow only, whilst figs. 1, *b* and 1, *c*, show that only this non-cartilaginous middle portion was used for explantation, the cuts at either end being well beyond the terminal masses of uneroded cartilage.

The rectangular bone fragments obtained from the middle parts of the tibiae were each explanted in a large hanging drop culture ($1\frac{1}{4}$ inch square coverslip over $3 \times 1\frac{1}{2}$ inch hollow-ground slide) in a semi-solid medium composed of equal parts of blood plasma and embryo extract. As previously described (Fell, 1932) the explants became surrounded by a zone of emigrating cells. On the third day of growth, the cultures were transferred to fresh medium and on the sixth day the original bone fragments were removed and rejected, as in my earlier experiments (Fell, 1932), and the ring of new growth was re-explanted into a medium consisting of about 2 parts of plasma and 1 part of embryo extract. After this stage the tissue was sub-cultivated every 48 hours, care being taken to preserve the central parts of the culture undamaged.

Sometimes the ordinary hanging drop method was modified, either by using an unusually thick clot, or by growing the tissue for a limited time in medium sandwiched between, and in contact with, two parallel glass surfaces. This was done with the object of increasing, if possible, the percentage of chondrogenic cultures. The results, however, were statistically inconclusive and will not be discussed in this communication.

A total of 29 cartilage-containing cultures were obtained out of over 190 endosteal explants. In the last experiment, to which I have already referred, fig. 1, out of 33 cultures examined by means of histological sections, 12 con-

tained cartilage, which was an unusually large proportion of chondrogenic growths.

The cultures were fixed in Zenker's solution containing 2-3 per cent. acetic acid, hardened with alcohol and decalcified with formol-nitric acid, using the method mentioned in my previous paper (Fell, 1932). Complete serial sections were cut of all the cultures studied, the plane of section always being parallel to that of the coverslip in the original culture. The sections were stained with eosin and thionin, with safranin and picro-indigo-carmin, or with Mallory's phosphotungstic acid hæmatoxylin, and were mounted in Gurr's neutral balsam.

Observations on the Living Material.

The original bone fragments were more or less rectangular and their longer sides, being cut at right angles to the Haversian canals, were somewhat jagged. It was usually possible to distinguish the Haversian canals as rather translucent bands running more or less parallel with the shorter sides of the rectangle.

After 16-24 hours' growth delicate spikes of protoplasm began to protrude from the openings of the Haversian canals. These represented the beginning of cell emigration. The rate at which the cells wandered out depended to some extent on the age of the chick from which the tibia was taken, and was rather more rapid for the 3-7 day birds than for the 13-day chick. Most of the cultures showed a rather scanty and irregular outgrowth after about 48 hours' cultivation, but by the end of the sixth day *in vitro*, that is, 3 days after transference to fresh medium, the bone had become surrounded by a broad halo of cells which, as previously described (Fell, 1932), usually showed two zones: an inner, compact region, and an outer zone composed of strands of cells radiating into the medium. In the cultures made from the younger chicks, the cells of the inner zone were often closely and fairly regularly arranged to form a continuous sheet, but in the cultures of 13-day bone the cells tended to be elongated in a direction parallel with the sides of the explants, and to be distributed in a chaotic manner like straw. A narrow belt of rather loose tissue often intervened between the bone and the inner, dense zone. The cells composing the greater part of the outgrowth were not individually distinguishable from fibroblasts but in the cultures of the younger bones they often differed from the latter in their habit of growth, since the compact, continuous sheets described above are not formed in ordinary fibroblastic growths. A variable number of wandering cells were almost always present.

In a few of the cultures derived from 3-6-day bones, osteogenic fibres were visible in the inner, compact zone of the outgrowth before the removal of the

bone fragment, but this was not observed in any of the cultures derived from the 7- or the 13-day tibiae. In all the experiments it was found that cartilage developed most readily when the zone of growth was completely undifferentiated when removed from the original explant. Cultures in which numerous bands of osteogenic fibres had developed in the outgrowth before removal of the bone seldom gave rise subsequently to cartilage. In several experiments in which a large number of endosteal cultures had been prepared, no example of chondrogenesis was obtained, and it was noted that this complete absence of cartilage was usually associated with partial differentiation of the outgrowths before the removal of the original bone. On the other hand, cartilage never appeared extensively in poorly growing cultures even if the zone of outgrowth was quite undifferentiated at the time of removal from the bone fragment, and such specimens almost always formed merely abnormal osteoid tissue.

When the original fragment had been excised on the sixth day of cultivation and the ring of outgrowth transferred to fresh medium, growth became extremely active and after 24-48 hours the central hole from which the bone had been taken was almost completely filled by the radial ingrowth of cells from the inner margin of the ring. Cells also emigrated rapidly from the outer margin and the entire ring gradually spread out into a sheet. Occasionally the tissue, after partially expanding in this way, suddenly became detached from the fibrin clot at one side, apparently owing to a local liquefaction of the medium, and shrank back into a small oblong mass lying to one side of a large circular area filled with fluid exuded from the retracted clot. After a further 12 hours' incubation these contracted cultures usually re-expanded to some extent, but they remained smaller and thicker than the rest.

The first signs of osteogenesis often appeared during the first 2 days following the removal of the bone, but the rate of differentiation varied in different cultures. As described in my previous paper the osteoblasts gave rise to compact masses of cells having an almost epithelial appearance and separated from each other by gelatinous partitions in which fine osteogenic fibres subsequently developed. Usually these partitions were extremely thin and inconspicuous, but in some cultures, especially in those which were rather dense and thick, they were in places much more strikingly developed so that the large, rounded osteoblasts appeared to be embedded in a homogeneous jelly which, though rather less refractile than cartilage matrix, gave the tissue a distinctly chondroid appearance. This structure was particularly striking in those cultures referred to above, in which the tissue had become detached from

the clot and contracted into a small mass during the first day's growth following the removal of the original bone.

The subsequent history of this gelatinous tissue varied. In some cultures osteogenic fibres appeared in the intercellular material, which eventually developed into ordinary bone matrix. In others, however, the intercellular substance in certain places remained homogeneous in appearance and became more refractile; at the same time the cells became spherical and rapidly enlarged, compressing the intercellular material which formed narrow capsules covering the cytoplasm. This tissue was unmistakable cartilage and even with low magnifications, fig. 2, *a*, Plate 19, was readily distinguishable from neighbouring osteoid tissue by its translucent, glistening appearance which contrasted strongly with the opaque, yellowish aspect of the osteoid regions. Chondrogenesis and osteogenesis usually took place simultaneously in the same culture, the developing cartilage and bone merging imperceptibly through a series of intermediate cell types. Sometimes the entire culture appeared cartilaginous with the exception of the marginal fringe of amœboid cells, but the commonest sites for chondrogenesis were in the newly formed tissue, which had grown into the central hole after the removal of the bone fragment, and in the new tissue immediately external to the original ring of growth; the original ring itself usually formed bone only. Chondrogenesis often took place with great rapidity so that a relatively large mass of cartilage was sometimes present 3 or 4 days after the removal of the tibial fragment.

If a culture, which at the third or fourth day after excision of the bone consisted largely or mainly of cartilage, was maintained for a longer period, the area of cartilage was seen to diminish in size and finally disappear, being replaced by fibrous, osteoid tissue which eventually developed into true calcified bone. Further study showed that this replacement of cartilage by osteoid tissue was effected by a direct transformation of the cartilage, the cells of which gradually decreased in size resuming the appearance of osteoblasts, whilst the matrix assumed the opaque, yellowish look characteristic of osteoid tissue.

Observations on Fixed Material.

A study of cultures fixed and sectioned at different stages of growth confirmed the observations made on the living tissue.

Six of the cultures of 13-day bone were fixed and sectioned after 6 days' growth, fig. 3, Plate 18, before the removal of the original fragment. The bone between the Haversian canals was considerably thicker than in the day-old chick tibia figured in my previous paper. As in my earlier experiments the

outgrowth appeared to be derived mainly, and probably exclusively, from the tissue in the canals, which in the normal tibia consists chiefly of thin walled blood vessels and a lining layer of osteoblasts (endosteum); there was no evidence that the bone cells participated. The canals in the explants contained capillaries enclosing degenerate erythrocytes, and numerous amoeboid osteoblasts many of which were in mitosis (*cf.* Studitsky, 1932). The outgrowth in the immediate neighbourhood of the bone was composed of rather loosely arranged, spindle-shaped cells, from which radiated the strands of amoeboid elements crawling into the plasma clot. It was not possible to distinguish with certainty the different histological types in the halo of outgrowth.

The first indications of chondrogenesis in the outgrowth after removal of the original bone-fragment, was well shown in several cultures, fig. 4. Areas occurred which consisted of more or less oval or amoeboid cells, in appearance identical with osteoblasts, these were embedded in an apparently homogeneous matrix. The preparations were stained with eosin and thionin, and in places the matrix was eosinophil as in early osteoid tissue, but elsewhere it was stained a pale sky-blue with thionin, displaying no affinity for the eosin. The next stage in development was usually seen in the same cultures. In certain areas the cells had enlarged and become almost completely spherical whilst the intercellular material stained a darker blue than in the less developed regions. At the margin of these chondroid areas the blue staining matrix merged into eosinophil material enclosing obvious osteoblasts.

The next stage in chondrogenesis was marked by the formation of a delicate capsule over the surface of the cytoplasm, fig. 5, Plate 19. This capsule stained a deep violet with thionin. As the chondroblasts continued to enlarge and to divide, the intercellular spaces became extremely narrow so that the gradually thickening capsules, fig. 6, Plate 20, were in contact with each other over most of their surfaces. The amount of cartilaginous matrix produced was relatively slight and consisted of little more than the rather thin cell capsules. In this the cartilage differed from that of the normal embryonic limb skeleton where even in early development the cells are separated by comparatively broad partitions.

Sometimes only a very small quantity of cartilage was present, and some cultures contained merely a tiny mass consisting of a few cells, or a few scattered, encapsulated chondroblasts lying in the osteoid tissue.

The cartilage formed in the endosteal cultures invariably developed rapidly into the hypertrophic type, fig. 7, Plate 20, similar to that in normal ossifying

cartilage, and never gave rise to the small-celled, non-ossifying form seen in the embryonic avian epiphyses and elsewhere. The chondroblasts in the cultures were usually neither so large nor so vacuolated as those of the late embryonic skeleton, and resembled rather the hypertrophic cells in a 7- or 8-day embryonic tibia or femur. This was probably owing to the fact that the cartilage in the cultures usually disappeared before it reached an advanced stage of development, and in order to obtain preparations containing cartilage it was therefore best to fix the cultures not later than 6-8 days after the removal of the original bone.

As described for the living cultures, the margin of a cartilaginous nodule was never clearly defined but merged gradually into the surrounding osteoid tissue through a belt of intermediate tissue, figs. 2, *b*, and 6. In this respect the histological structure differed strikingly from that of the normal embryonic limb skeleton where the boundary between the cartilage and the surrounding bone is extremely distinct. The cells in this transitional tissue varied in appearance. A few, though not encapsulated, resembled chondroblasts, being large, round and vacuolated, others possessed a thionin staining capsule but otherwise resembled osteoblasts, whilst the majority, some of which were encapsulated with a film of cartilaginous matrix, were intermediate in appearance between osteoblasts and chondroblasts. The intercellular material was fairly plentiful and, with the exception of the cartilaginous capsules, was almost colourless in preparations stained with thionin and eosin.

As already described, osteogenesis and chondrogenesis proceeded simultaneously in the same culture and the intermediate tissue originally marked the line of junction of the chondrifying and ossifying areas, fig. 8, Plate 20. As the age of the culture advanced the intermediate zone gradually encroached internally on the cartilaginous area whilst externally it became transformed into osteoid tissue and eventually bone. Thus the region of cartilage, as already stated, became progressively smaller. Sections of cultures in which a mass of cartilage had been seen to diminish in size and apparently disappear during life, sometimes showed a little typical cartilage in the middle of the original cartilaginous area which elsewhere consisted of intermediate tissue merging with osteoid tissue. In other cultures typical cartilage with thionin staining matrix had completely disappeared, and the area was composed entirely of intermediate tissue, fig. 9, Plate 20, with colourless or faintly eosinophil intercellular material. Some of the cells in such areas were still enclosed in thin capsules which, however, no longer gave the typical meta-chromatic staining reaction with thionin.

The histological preparations indicated that the cartilage at any period of its development might be directly transformed into osteoid tissue but that the change took place most rapidly in the early stages of chondrogenesis. Sometimes an early chondroid area might be almost completely converted into osteoid tissue in about 48 hours, but in others the centre of the cartilage had time to reach a more advanced differentiation so that the transformation was checked in this central, better differentiated region, and proceeded more slowly than at the periphery.

Discussion.

No cartilage was present in the original bone fragments, so that the first question which arises is whether the cartilage, which sometimes appeared in the endosteal cultures, was formed by osteoblasts, or whether it was formed by chondroblasts, which had been liberated by the erosion of the original cartilage rudiment, and had then either remained adherent to the inner surface of the tube of periosteal bone or had wandered outwards into the Haversian canals. On the assumption that the cartilage must have been derived from latent chondroblasts from the cartilaginous skeleton, we should have expected a sharp demarcation between the cartilage and the surrounding bone as in normal development, and it would be difficult to account for the intermediate type of tissue, which always intervened between the cartilaginous and osteoid areas, forming such a perfect gradation between the two tissues. It seems more probable that the osteoblasts of the Haversian spaces, as a result of their active proliferation and migration, dedifferentiated into an indifferent type of skeletogenous cell capable of producing either cartilage or bone according to the environmental conditions. This is supported by the fact that, as described above, the first stage in the development of either cartilage or bone in endosteal cultures was the formation of a primitive tissue composed of osteoblast-like cells embedded in a gelatinous matrix, and it was at first impossible to foretell whether such tissue would eventually prove to be chondrogenic or osteogenic. Ham (1930), in an interesting paper on the repair of experimental fractures, is of the opinion that "the osteogenic cells cannot be called osteoblasts or chondroblasts," a view which is confirmed by the results described in the present communication.

It was interesting to note that the chondroblasts in the endosteal cultures were always the hypertrophic type so characteristic of ossification centres in the normal embryonic skeleton. The work of Robison and his colleagues (Robison and Soames, 1924; Martland and Robison, 1924) has afforded evidence that, like osteoblasts, these hypertrophic cartilage cells secrete a phosphatase

which is not synthesized by the smaller cells of non-ossifying cartilage. The results recorded in this paper suggest that a very close histogenetic and physiological relationship exists between osteoblasts and hypertrophic chondroblasts.

The unstable character of the cartilage in the endosteal growths was very striking. The view that bone can develop by the direct transformation of cartilage has often been expressed, and it must be admitted that I have hitherto regarded such statements with scepticism. There is no doubt that no such transformation occurs in the normal histogenesis of the long-bones of the fowl, where the cartilage is cleanly excavated from within the tube of periosteal bone. Nevertheless a study of chondrogenic endosteal cultures both in life and by means of histological preparations has forced me to the conclusion that a direct transformation of cartilage into bone actually occurs in this material. As already described, the cartilage formed in endosteal growths differed markedly from that of the normal limb skeleton in the much smaller quantity of intercellular material present even in the most highly developed cultures obtained. In most cultures the typical, thionin staining matrix consisted of little more than relatively thin capsules enclosing the cells. It is possible that this comparatively low differentiation of the cartilage may explain its capacity for direct transformation into bone, and it is probable that such direct transformation would become impossible if a more advanced degree of differentiation were attained.

The cartilage of the endosteal cultures bore a remarkably strong resemblance to some of the cartilaginous areas, which are not uncommonly found in healing fractures, and the latter show the same intermediate tissue, which was so conspicuous in the cultures, intervening between the cartilage and surrounding bone. Professor H. M. Turnbull most kindly allowed me to examine some of his preparations of human fractures, and the histological picture in the cartilaginous parts of the human callus and in the endosteal cultures was sometimes almost indistinguishable.

The factors responsible for the differentiation of cartilage in a fractured bone may be identical with those which induce chondrogenesis in the endosteal cultures. As regards the endosteal cultures, the results described above provide no information concerning the immediate causes of chondrogenesis. If these causes could be defined much light might be shed on the physiological conditions involved in the differentiation of the original cartilaginous skeleton, a problem of so much importance from the evolutionary standpoint.

The fact that endosteum can develop into both cartilage and bone under the

simplified conditions obtaining *in vitro* makes the prospect of determining the external factors concerned considerably more hopeful. This aspect of the subject is now being investigated.

I wish to express my gratitude to Dr. J. S. F. Niven for valuable criticism.

The expenses of the investigation were defrayed by the Medical Research Council.

Summary of Results.

(1) Cartilage develops in a minority of endosteal cultures and is always associated with bone or osteoid tissue.

(2) The cartilage merges gradually into the surrounding osteoid tissue through a belt of intermediate tissue.

(3) The cartilage cells are always of the hypertrophic type characteristic of ossification centres in the normal embryonic skeleton.

(4) The cartilage differs from that of the normal skeleton in the comparatively low stage of differentiation which it attains, as indicated by the relatively small quantity of matrix formed.

(5) The cartilage is unstable and, at least in most cases, becomes directly transformed into osteoid tissue and bone.

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DESCRIPTION OF PLATES.

FIGS. 1 *a*, 1 *b*, 1 *c*, 2 *a*, and 2 *b*, are photomicrographs, the other figures are drawings made with the aid of a projection prism. All the sections of cultures illustrated were cut parallel with the plane of the coverslip of the original culture-vessel. Photomicrographs by Mr. V. C. Norfield.

List of Abbreviations.

b., bone; *c.*, cartilage; *ca.*, capsule; *Ha.c.*, Haversian canal; *i.t.*, intermediate tissue; *m.*, medium; *ma.*, marrow; *os.*, osteoid tissue; *z.o.*, zone of outgrowth.

PLATE 18.

FIG. 1.—*a*. Longitudinal section through the tibia of a 7-day (hatched) chick. The cartilage has been completely eroded from the middle region of the shaft which consists of

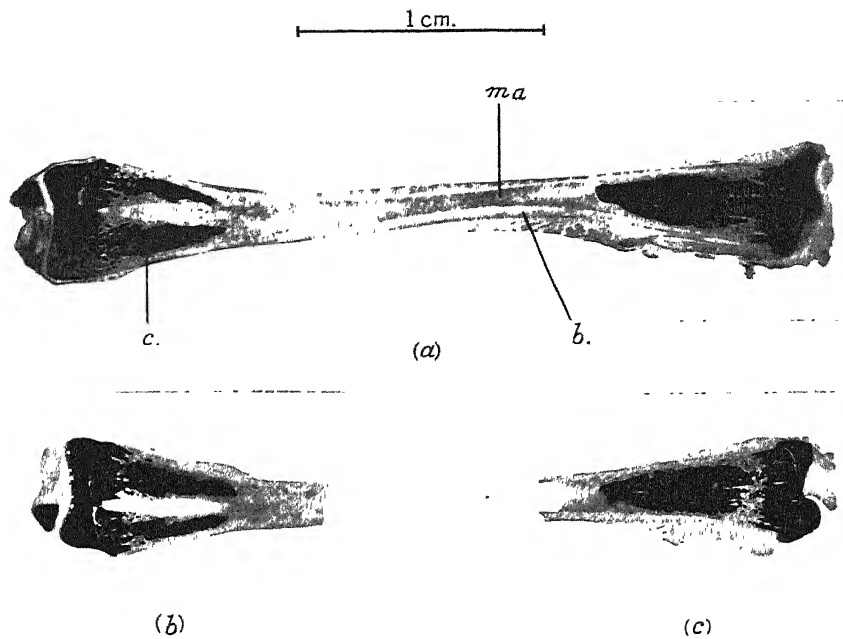


FIG. 1.

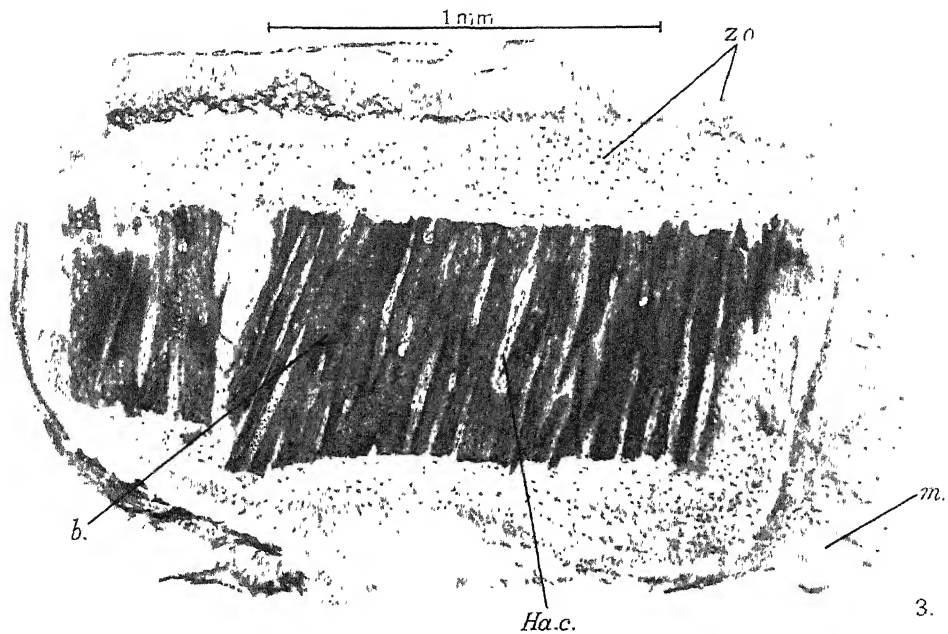


FIG. 3.

H. B. F. del.

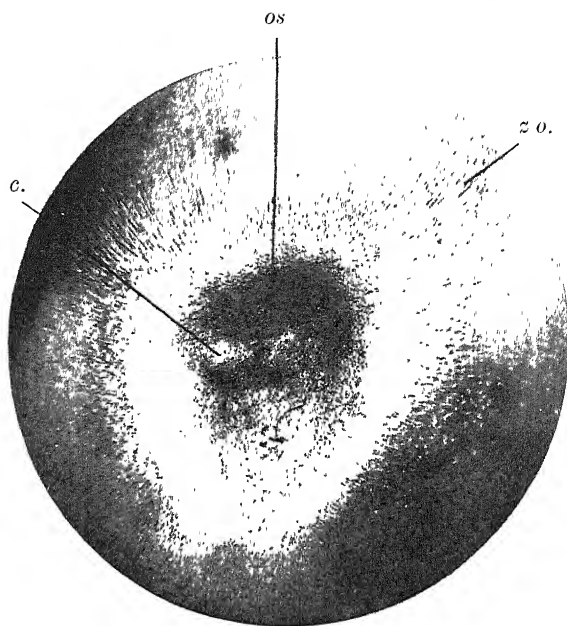


FIG. 2A.

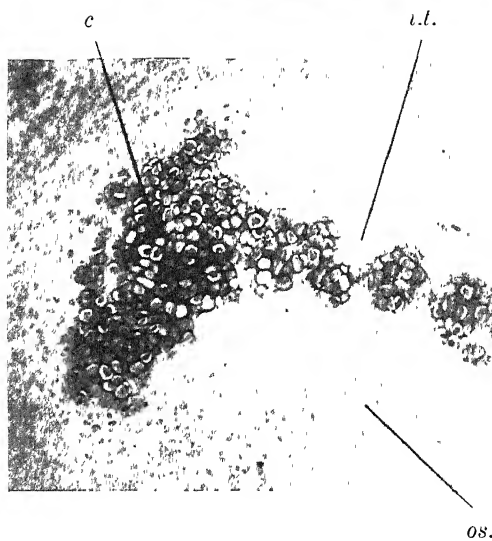
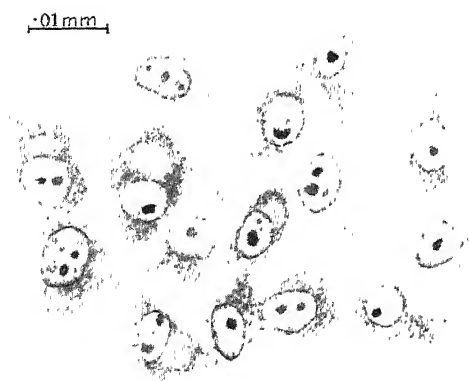
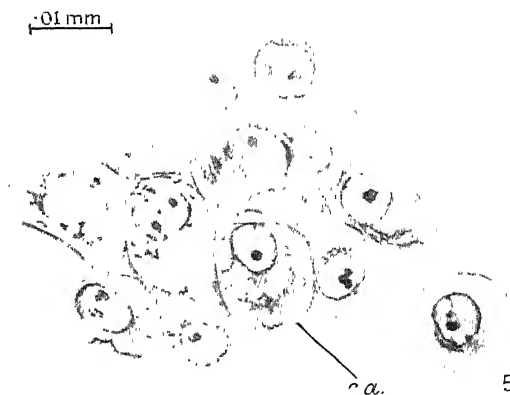


FIG. 2B.



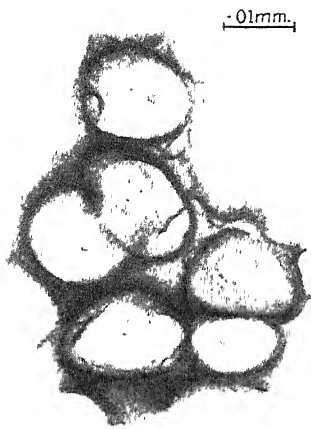
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FIG. 4.



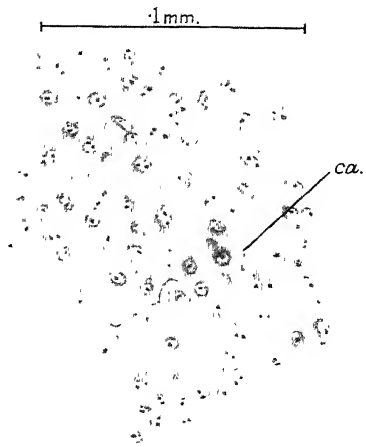
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FIG. 5.



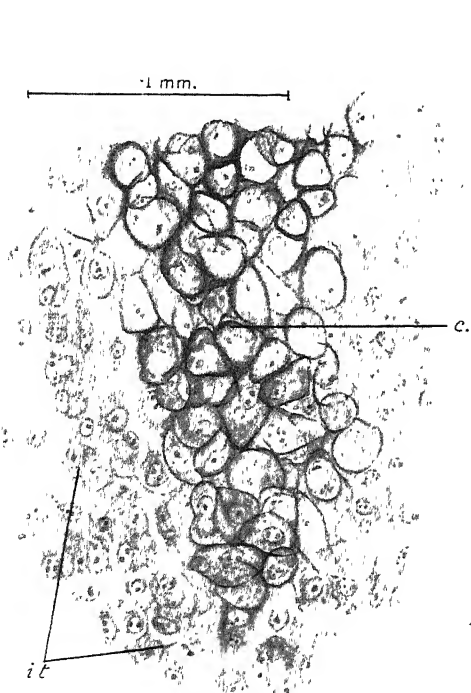
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FIG. 7.



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FIG. 9.



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FIG. 6.



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FIG. 8.

a tube of periosteal bone containing marrow. *b* and *c*. Longitudinal sections of the cartilaginous ends of the tibia from the opposite side of the same 7-day chick. The non-cartilaginous middle region of this tibia was cut out, stripped of periosteum and cut up into small explants. Twelve chondrogenic cultures were obtained from this bone. (Eosin, thionin.)

FIG. 3.—Section through a 6-day culture of bone from the tibia of a 13-day chick, fixed at the stage immediately prior to the removal of the original bone fragment. Note the broad halo of emigrated cells surrounding the bone. (Mallory's phosphotungstic acid hæmatoxylin.)

PLATE 19.

FIG. 2, *a*.—Living culture of endosteum derived from the tibia of a 6-day chick, photographed 6 days after removal of the original bone fragment and immediately before fixation. The interior of the culture contains an elongated nodule of cartilage surrounded by osteoid tissue and bone.

FIG. 2, *b*.—Section of same culture showing the cartilage. Note the zone of intermediate tissue intervening between the cartilage and the surrounding osteoid tissue. (Eosin, thionin.)

FIG. 4.—Section of an endosteal culture derived from the same tibia as the culture shown in fig. 2, and fixed 6 days after the removal of the original bone. The intercellular material in the area figured had stained a pale blue with thionin and probably represented the first stage in chondrogenesis. The osteoblast-like cells are not encapsulated. (Eosin, thionin.)

FIG. 5.—Section of an endosteal culture derived from the same tibia as the culture shown in fig. 2, and fixed 4 days after the removal of the original bone. Young chondroblasts are seen which have become enclosed by a delicate capsule giving the characteristic metachromatic, violet stain with thionin. (Eosin, thionin.)

PLATE 20.

FIG. 6.—Section of an endosteal culture derived from the tibia of a 3-day chick and fixed 6 days after the removal of the original bone. This specimen shows part of a nodule of cartilage surrounded by a zone of intermediate tissue which externally passes into osteoid tissue (not shown in the figure). (Eosin, thionin.)

FIG. 7.—Section of an endosteal culture derived from the tibia of a 4-day chick and fixed 8 days after the removal of the original bone. This was part of the most highly developed area of cartilage obtained, but nevertheless a comparatively small quantity of matrix has been formed. Note the vacuolated, hypertrophic chondroblasts within the capsules. (Eosin, thionin.)

FIG. 8.—Section of an endosteal culture derived from the tibia of a 6-day chick and fixed 3 days after the removal of the original bone. Note the osteoid tissue merging gradually with a large cartilaginous area. (Safranin, picro-indigo-carmin.)

FIG. 9.—Section of the same culture as that shown in fig. 2, *b*. This figure shows an area in which the transformation of cartilage into osteoid tissue is almost complete. Most of the chondroblasts have assumed an osteoblastic appearance and the capsules were eosinophil only and no longer displayed the metachromatic staining with thionin. (Eosin, thionin.)

*The Luminous Efficiency of Rays Entering the Eye Pupil
at Different Points.*

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(Communicated by Sir John Parsons, F.R.S.—Received December 15, 1932.)

1. *Introduction.*

The effect to be described in this paper was discovered in attempting to develop an apparatus for measuring the area of the eye pupil, using a photometric principle. The idea involved is made clear by the diagram fig. 1. The subject applies his eye to the eye-ring E and fixes his eye on the aperture

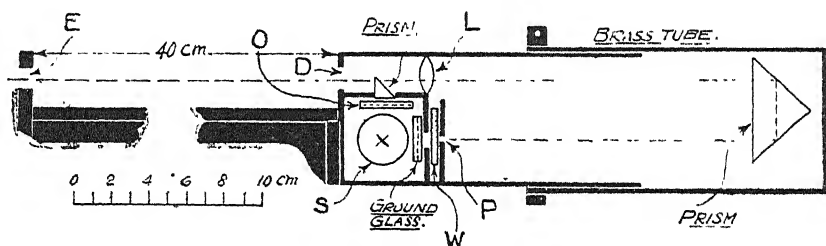


FIG. 1.—Photometric pupillometer.

in the diaphragm D. He then sees a photometric field divided into two parts. The lower half is illuminated by light diffused from the opal O, which itself receives light from the lamp S. The rays from this half of the field form a diverging beam which completely fills the pupil of the subject's eye. The upper half of the field is illuminated by the method of Maxwellian view. An image of the small aperture P is focussed by the lens L on to the middle point of the eye-ring E. This image is only about 1.5 mm. in diameter and all the light is collected by the subject's eye, provided the pupil of the latter is concentric with the eye-ring and has a diameter exceeding 1.5 mm. A variable graduated neutral wedge W is inserted at the aperture P and this enables the two halves of the photometric field to be adjusted to equality of brightness by the subject.

According to the usual assumption that the apparent brightness of an object viewed in the normal way is proportional to the pupil area, the brightness of the lower half of the field will be proportional to the pupil area A. The brightness of the upper half of the field, on the other hand, is independent

of the pupil area. Thus if the fields match for a setting of the wedge corresponding to a transmission t , then A will be proportional to t ; $A = \alpha t$. The constant α is determined by an observation with an artificial pupil of 3 mm. diameter.

When tests were made with this apparatus anomalous results were obtained. It appeared that even when the pupil was fully extended and had a diameter of about 8 mm., the value derived by the above method never exceeded 5.5 mm. After all conceivable instrumental errors had been investigated it was concluded that the discrepancy was due to the fact that the apparent brightness of an object is not proportional to the pupil area because the rays entering the pupil at points distant from the axis are not so effective visually as rays entering along or near to the axis. The quantitative study of the effect was then undertaken, and for this purpose the pupillometer shown in fig. 1 was modified, a Maxwellian field being used for both beams. An extended series of observations was made with the modified apparatus, but it was finally decided to construct a new apparatus incorporating certain necessary improvements. As the earlier measurements are in good general agreement with those obtained with the new apparatus, only the latter need be communicated.

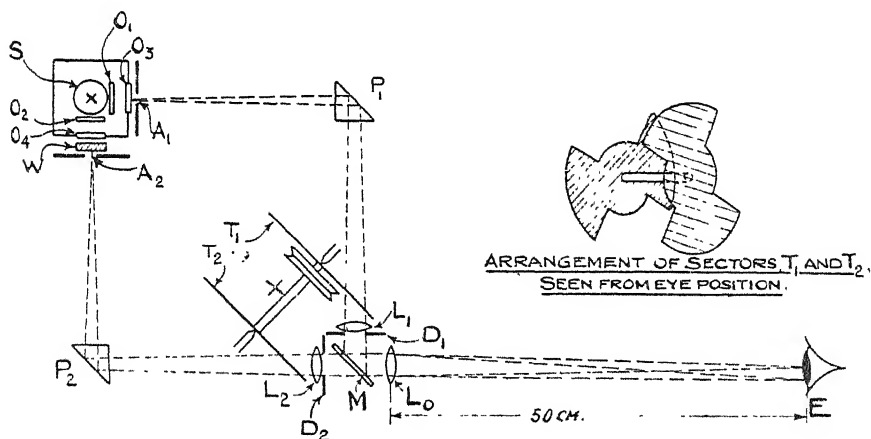


FIG. 2.—Apparatus for measuring the luminous efficiency of light rays entering the eye pupil at different points.

2. *Description of the Apparatus and Method.*

A diagram of the apparatus is shown in fig. 2. The apparatus can be used to make a photometric match either by the equality of brightness method, or by the flicker method. For the present work the flicker method presents advantages, and it is this form which will be described. The light source S

(a 100-volt 100-watt tubular projection lamp run at 60 volts to obtain a suitable field brightness) is contained in a metal box and illuminates two windows of diffusing glass O_1 , O_2 . These in turn illuminate two other windows of diffusing glass O_3 and O_4 (the double diffusion is adopted to obtain in the planes of the apertures A_1 and A_2 a closer approximation to the brightness of a perfect diffuser uniformly illuminated). Consider, to begin with, the "fixed" beam which originates from the small aperture A_1 . The beam is turned through a right angle by the prism P_1 and is rendered parallel by the lens L_1 . The parallel beam is reflected at the plain glass mirror M and is focussed to form an image I_1 in the plane of the pupil of the subject's eye, by the lens L_0 . Owing to the corneal refraction the actual material pupil differs in size and position from the apparent pupil seen from outside the eye. Here and elsewhere in the paper it will always be the apparent pupil which is referred to when the pupil area, diameter, position, etc., are specified. The eye, of course, does not see the image I_1 . The method of Maxwellian view is being used and what the eye sees is the lens L_0 (or L_1) illuminated to a uniform brightness. Actually two beams are formed by reflection from the two faces of the plane glass mirror, but by making the latter slightly wedge-shaped the image formed by one of the beams is shifted well outside the subject's eye. This beam is then not seen at all.

Turning now to the "traversing" beam, this starts from the small aperture A_2 , is turned through 90° by the prism P_2 , is rendered parallel by the lens L_2 , traverses the plane glass and again is finally focussed to form an image I_2 in the plane of the pupil of the subject's eye. The light forming the traversing beam is made to pass through the graduated neutral absorbing wedge W .

The prism P_1 is fixed in a support with adjusting screws which enable the image I_1 to be brought to a suitable position in the plane of the pupil. A more elaborate mounting is adopted for the prism P_2 , as by rotations of this prism about two axes at right angles, the image I_2 of the traversing beam can be made to enter the eye pupil at any desired point. P_2 is first mounted upon a small table with adjusting screws, and the table is mounted at the pivot end of a lever 18 cm. long (axis of rotation AB in fig. 3). This lever is in turn mounted on a second lever whose axis of rotation CD is at right angles to the first. AB and CD intersect at the centre of the reflecting face of the prism. Both levers can be tilted through small angles by tangent screws acting against their ends. The screws are 1 mm. pitch with heads divided into 100 parts; tenths of a division can be estimated. This gives an accuracy of location of the traversing image I_2 of 0.003 mm. for the horizontal direction and

0.0015 mm. for the vertical direction; an accuracy of 0.01 mm. is sufficient in practice.

In making a photometric match by the flicker method, the brightnesses to be compared have to be presented alternately at a rapid rate of alternation. The brightnesses are then adjusted so that there is no sensation of flicker. The alternation is accomplished in the present apparatus by the two sector disks T_1 , T_2 mounted on a common axle X. Each sector disk comprises two

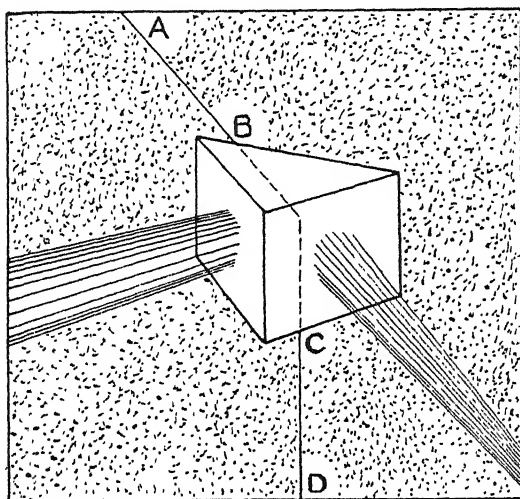


FIG. 3.

open and two closed quadrants in alternation, and the disks are mounted, on the axle X, 90° out of phase so that as the fixed beam is disclosed the traversing beam is occulted.

The field as seen by the subject is delimited by the identical circular apertures D_1 (fixed beam) or D_2 (traversing beam). The angular diameter of the field equals 1° .

It was essential for these measurements to have the subject's eye in a fixed and reproducible position. This was accomplished by requiring the subject to bite on to a small brass plate thickly coated with sealing wax. This was done with the wax softened by heat but on the point of solidification. On cooling the wax, a good impression of the teeth was obtained. The brass plate was then fixed rigidly to the apparatus in such a position that when the subject fitted his teeth into the wax impression and fixed his eye steadily on to the mid-point of the lens L_0 , his eye was at the right position for observation. This

method was very successful in giving a precise and reproducible positioning of the subject's eye.

The principle of the measurements is now briefly as follows. The images I_1 and I_2 are both adjusted to coincide with the centre point of the subject's pupil. The sector disks are set running and the subject adjusts the wedge W so that the field ceases to flicker. If the sector speed is too high there is a wide range of wedge settings over which the field is flickerless. If the sector speed is too low, it becomes impossible to eliminate flicker. The motor driving the sectors is in the control of the subject who soon finds the most suitable speed at which to work. When a set of six settings has been made, the image I_2 is shifted to a new position, say a millimetre to the right of I_1 , and the measurement is repeated, and so on. If the luminous efficiency of the light were independent of its point of entry in the pupil opening, the wedge readings would all be the same. Actually there is a steady change of wedge reading as the image I_2 is shifted out from the axis to the periphery of the pupil.

The following special points must be briefly dealt with.

Calibration of the Traversing Image I_2 .—This was accomplished by erecting a travelling microscope to view the image I_2 . The displacement of I_2 in the vertical (or horizontal) direction for a given turn of the tangent screw was determined. To a sufficient accuracy the displacement of the image could in each case be taken as proportional to the amount of rotation of the screw.

Dilatation of the Pupil.—To enable the measurements to be made with the image I_2 as far from the axis of the eye as possible, the subject's pupil was dilated using the mydriatic, euphthalmine hydrochloride. About four drops of a 5 per cent. solution sufficed to keep the pupil dilated throughout the day. This mydriatic has very little effect on accommodation.*

Centring of the Image I_2 .—The wax-covered mouthpiece bearing the impression of the subject's teeth having been adjusted and fixed so that, with the tangent screws in their mean positions, the image I_2 entered the eye approximately at the mid-point of the pupil, the exact position of the pupil centre was determined in the following manner. The horizontal traversing screw was turned until the subject saw the photometric field go dark, which occurred when the image I_2 passed across the edge of the pupil. (For this purpose the sector disks T_1 and T_2 were stationary and in a position to transmit the traversing beam.) A similar setting was made for the passage of I_2 across the opposite edge of the pupil. The mean of these two settings

* The authors' thanks are due to Dr. W. S. Duke-Elder for advice on the use of mydriatics.

gave the mid-point of the pupil as regards the horizontal direction. A similar method was used for determining the mid-point in the vertical direction.

Adjustment of the Apertures D_1 and D_2 .—With I_2 set at the mid-point of the pupil, the adjusting screws on the prism P_1 enabled I_1 to be brought into coincidence with I_2 . The apertures D_1 and D_2 were then adjusted so that the circular fields of the fixed and traversing beams, as seen by the subject, were exactly superposed. When, however, the image I_2 was displaced so that it no longer coincided with I_1 , then, owing to the aberrations of the optical system of the eye, the two fields were in general no longer coincident. Thus for every position of the image I_2 it was necessary to readjust the apertures D_1 and D_2 to obtain the desired superposition of the fields. When the readjustment entailed relative shift of D_1 and D_2 in the vertical direction, it was also necessary to change slightly the phase difference of the sector disks T_1 and T_2 . If a single aperture at L_0 had been used to delimit the field no compensation for the eye aberrations would have been possible.*

Calibration of the Wedge.—The absolute transmission of the wedge was not required but only the relative transmission at different settings. The wedge reading corresponding to a photometric match of the fixed and traversing beams was determined by the equality of brightness method with various sector disks of known transmissions inserted in one or other of the beams. The other conditions were kept unchanged during these measurements, *i.e.*, the images I_1 and I_2 remained coincident at the centre of the pupil, and so on. If t is the transmission of the wedge corresponding to a wedge reading r , then the calibration measurements can be used to draw a curve of kt against r , where k is an unknown constant whose value need not concern us. It is more convenient to work with the optical density of the wedge, $d = \log_{10} I/t$, and the calibration curve then becomes $(d + K)$ against r where again K is an unknown constant. This calibration curve was found to approximate to a straight line, with the wedge used.

3. *The Main Measurements.*

It is not yet known definitely whether the condition of adaptation of the eye and the brightness of the photometric field and its colour, will modify the effect, but a few preliminary observations on these points suggest that any

* The relative shifts of apertures D_1 and D_2 can readily be measured, and it is possible that such measurements would provide useful data regarding the aberrations of the eye. As far as the authors know such a method of investigating eye aberrations has not previously been used.

such modifications will be small. The actual conditions for the main measurements were the following :—

Apparent Brightness of the Photometric Field.—The photometric field as seen by the subject had an apparent brightness equal to that of an external brightness $B = 0.60$ c./sq. ft. viewed, in the normal manner, through an artificial pupil of 3 mm. diameter.

Colour of the Photometric Field.—This was practically the colour of the light emitted by the source. The colour temperature of a 100-volt 100-watt tubular projection lamp when run at 60 volts, is known from measurements made at the N.P.L. and equals approximately 2300° absolute.

Adaptation of the Eyes.—The subject wore an eye shade over the unused eye. The measurements were made in a darkened room.

Position of the Retinal Image.—Since the subject fixed his eye on the 1° photometric field the observations refer to the fovea centralis.

Size of the Images I_1 and I_2 .—The images I_1 and I_2 were circular and of diameter 0.75 mm. (I_1), 0.5 mm. (I_2).

Horizontal and Vertical Traverses.—Starting with the images I_1 and I_2 coincident two sets of measurements were made, (i) the horizontal traverse in which I_2 is moved step by step across the pupil opening on a horizontal line through the pupil centre and (ii) the vertical traverse, analogously defined.

Six settings of the wedge were made and the mean of the six taken as the reading for any one match. Suppose r_0 is the reading when the images I_1 and I_2 are coincident and r_x is the reading for any other position X of I_2 . The relative luminous efficiency η of a ray entering the pupil at the position X is defined as the ratio of the intensity of a ray incident axially to that of a ray incident at X and producing an image of the same apparent brightness. Thus,

$$\eta = \frac{t_0}{t_x} = \frac{(kt_0)}{(kt_x)}, \quad (1)$$

where t_0 and t_x are the transmissions corresponding to settings r_0 and r_x of the wedge. The values (kt_0) and (kt_x) are obtained directly from the calibration data.

In figs. 4 to 9 the mean values of η obtained from two or three independent sets of measurements, are plotted against the separation d in millimetres between the images I_1 and I_2 (I_1 is always fixed and situated at the centre of the eye pupil).

An examination of these curves, which have the same general shape both for the horizontal and vertical traverses and for the three subjects, shows that

the luminous efficiency tends to decrease as the point of entry moves away from the pupil centre and falls to a value of the order of one-third, at points near the periphery of the dilated pupil. Each curve is approximately a symmetrical curve though the peak does not always coincide in position with the centre of the pupil.

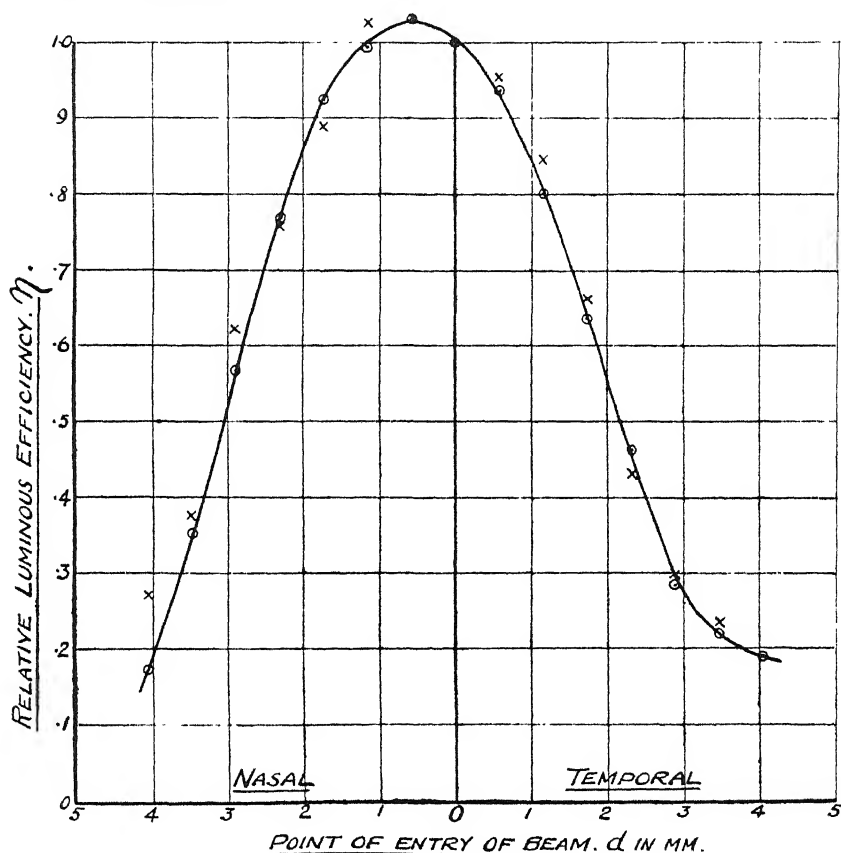


FIG. 4.—Horizontal traverse, subject B.H.C. ○ right eye; × left eye.

For the subject B.H.C. traverses for both eyes were obtained. As figs. 4 and 5 show, the shapes of the curves were the same, but the peak, although displaced to the nasal side in both eyes, is differently placed in the vertical traverses.

The horizontal traverse for the subject F.W.C., fig. 6, terminates at a much lower value on the nasal side than on the temporal side. It was noted that the pupil opening for this subject was appreciably eccentric with respect to the outer edge of the iris.

For the subject W.S.S. the curves were redetermined after an interval of about 6 weeks and showed a significant difference of shape, figs. 8 and 9. The measurements for B.H.C. were also repeated but no significant change could be detected.

All the above measurements were made using the flicker method. It was felt desirable to check the results against measurements by the equality of

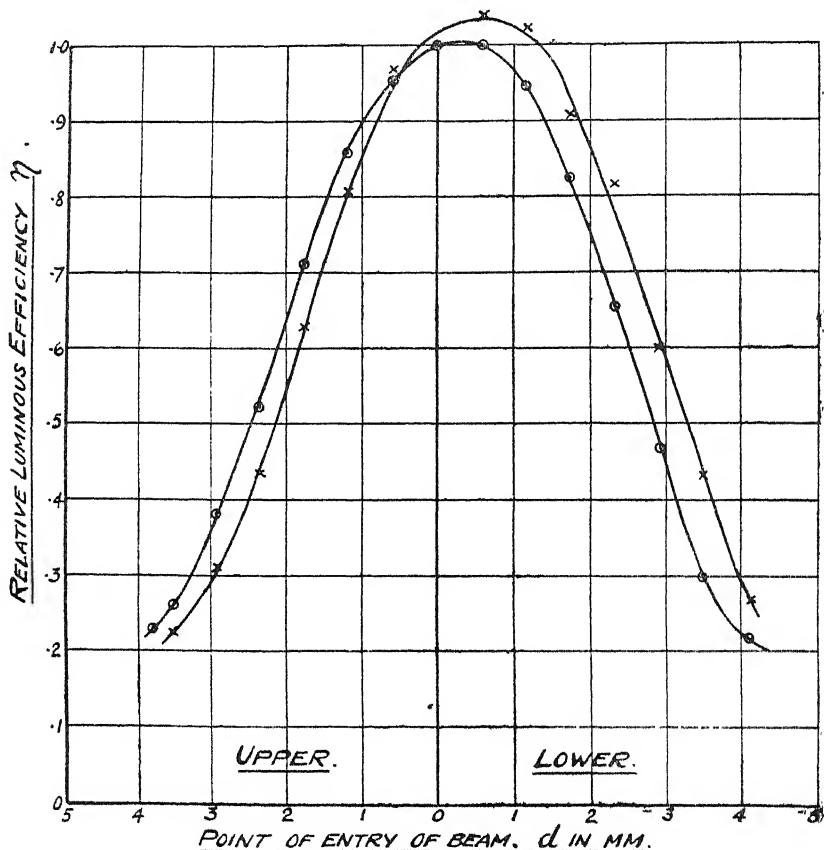


FIG. 5.—Vertical traverse, subject B.H.C. ○ right eye; × left eye.

brightness method. The apparatus was therefore used with the sector disks T_1 and T_2 fixed in such a position that the subject saw a bipartite photometric matching field. A comparison of a curve obtained by this method with the mean curve obtained by the flicker method shows that there is no significant difference between the results, fig. 10. The flicker method was preferred because imperfections in the field illuminated by the traversing spot, which

begin to come in when I_2 is about 3 mm. from the centre, are less disturbing than in the equality of brightness method.

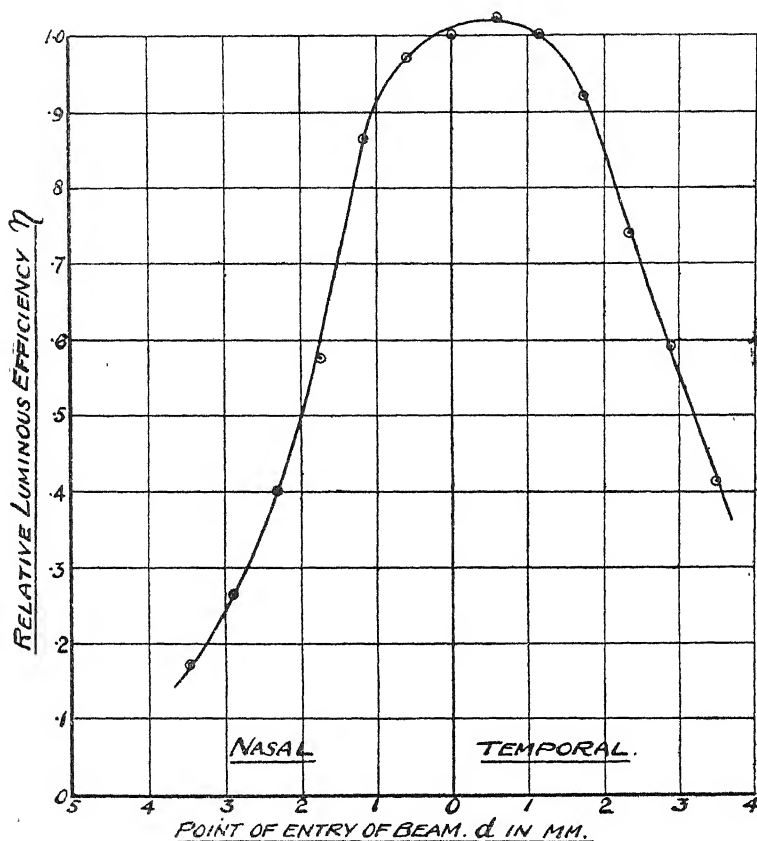


FIG. 6.—Horizontal traverse, subject F.W.C. Right eye.

4. Integrated Effect for Large Entrance Pupils.

If the value of η is known for each point of entry in the pupil opening, we can determine, by integration, the relative luminous efficiency for an entrance pupil at the eye of any size or shape. If the position of a point in the pupil opening is specified by polar co-ordinates r , θ , taking the origin at the centre of the pupil and measuring θ from the upward vertical, then for any given entrance pupil the relative luminous efficiency is given by

$$E = \frac{\iint \eta(r, \theta) r \sin \theta \, d\theta \, dr}{\iint r \sin \theta \, d\theta \, dr} \quad (2)$$

where the integrals extend over the whole area of the entrance pupil. The case of most practical interest is when the entrance pupil is circular and concentric with the pupil opening. The actual iris when it is allowed to react normally forms such an entrance pupil, the diameter varying with the brightness level in the field. Using the values of η given in the previous section calculations were made of the relative luminous efficiency E for circular entrance

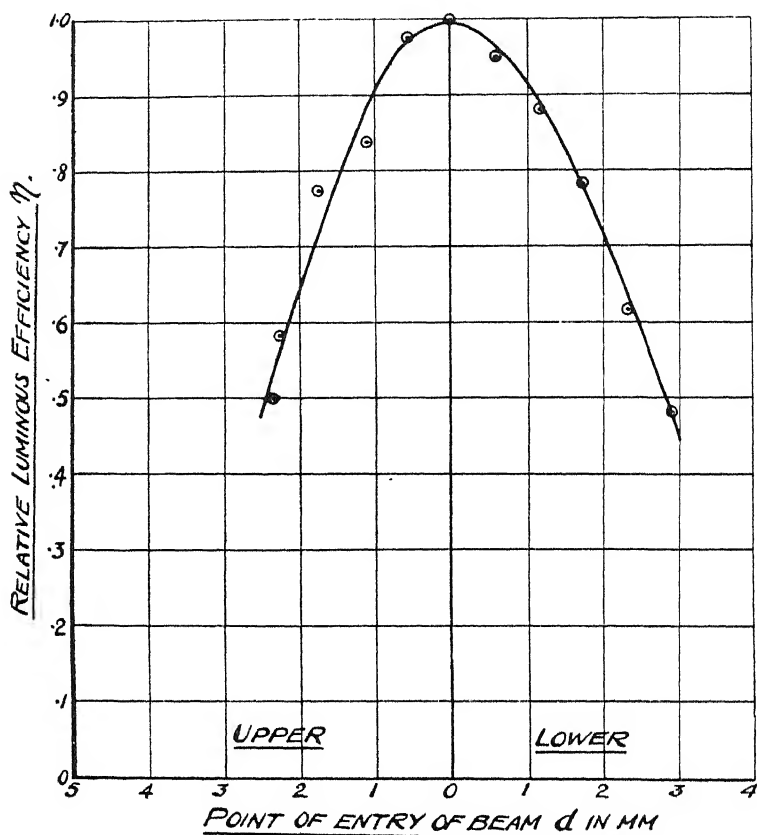


FIG. 7.—Vertical traverse, subject F.W.C. Right eye.

pupils of different diameters δ , and the results are plotted as the continuous curves in figs. 11 to 13, which give $\log_{10} E$ as a function of the area a of the entrance pupil. For the purposes of the calculation, it has been assumed that the efficiency $\eta(r, \theta)$ depends only on the distance away of the point of entry (r, θ) from a fixed point in the pupil, (r_0, θ_0) , which is near to the pupil centre but does not quite coincide with it. This assumption is consistent with the experimental result that the horizontal and vertical traverses give almost

identical symmetrical curves with the peaks slightly displaced from the centre. The position of the point (r_0, θ_0) is determined immediately from the displacements of the peak in the horizontal and vertical traverses. The integrations involved in equation (2) were performed graphically.

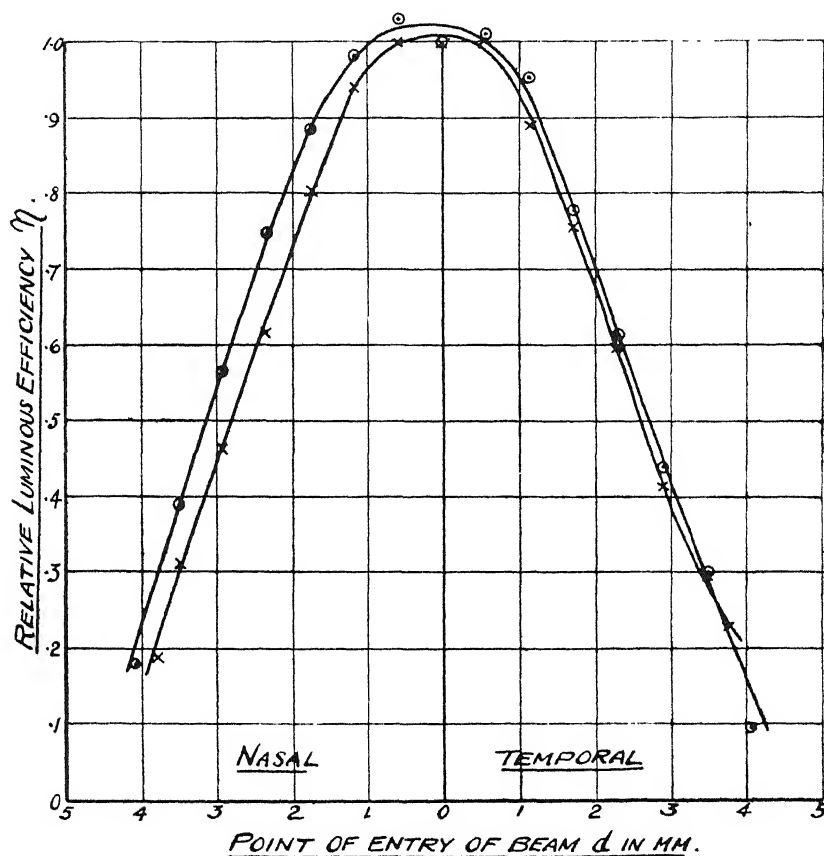


FIG. 8.—Horizontal traverse, subject W.S.S. \odot Original measurements; \times six weeks later.

It is apparent from the continuous curves of figs. 11 to 13, that $\log_{10} E$ as computed varies very nearly linearly with a . We have, approximately, $\log_{10} E = -ga$, where g is a constant.

Direct experimental determinations of E were obtained by the following method. Referring to fig. 2 the aperture A_1 , which had previously been kept the same in all the measurements, was replaced in turn by a series of circular apertures B_1, B_2 , etc., of increasing diameter. On the other hand, the image I_2 was now set permanently at the centre point of the pupil. Each of the

apertures B_n when inserted was adjusted so that its image J_n was concentric with I_2 and the pupil opening. The diameters of the images J_n were determined by direct observation using the travelling microscope. A photometric match was made with each of the apertures B_n . It is clear that if the brightness of the images J_n is uniform and the same for all, then the corresponding relative luminous efficiency E_n is proportional to t_n/A_n or to $(kt_n)/(\delta_n)^2$ where (kt_n)

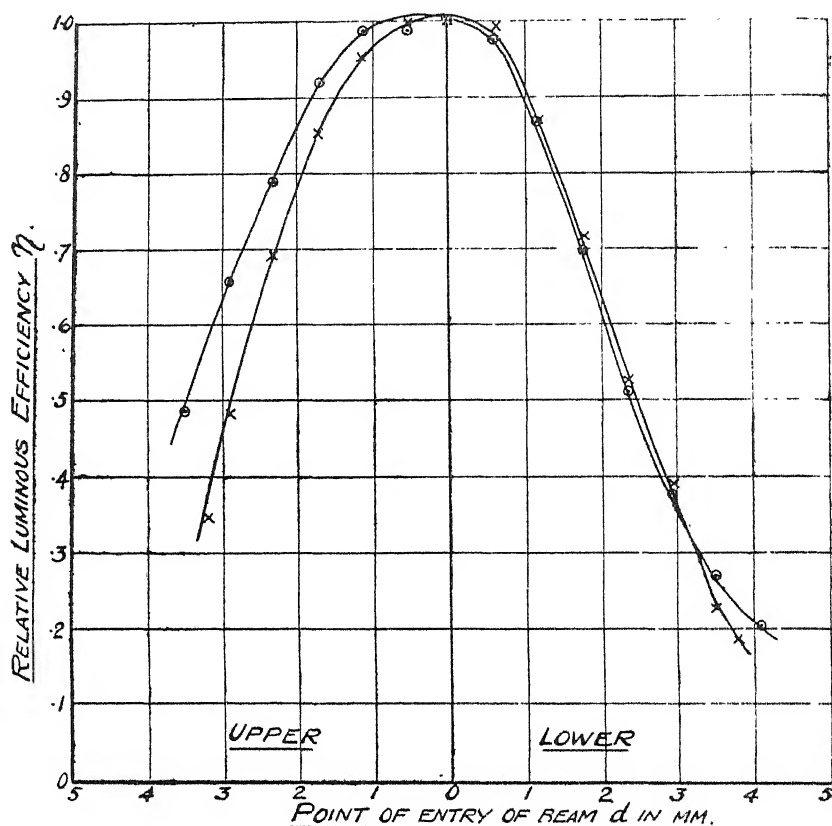


FIG. 9.—Vertical traverse, subject W.S.S. ○ Original measurements; × six weeks later.

is the mean wedge setting with the aperture B_n , which produces an image J_n of area a_n , and diameter δ_n . The constant of proportionality depends on the size and brightness of the image I_1 and is unimportant. In practice, the values of $(kt_n)/(\delta_n)^2$ were determined for a series of twelve apertures B_n giving values of δ_n ranging from 0.75 to 6.25 mm. Since the relative luminous efficiency E_n is proportional to $(kt_n)/(\delta_n)^2$, we have

$$\log_{10} E_n = \log_{10} \frac{(kt_n)}{\delta_n^2} + \text{a constant.} \quad (3)$$

The value of the constant was adjusted so as to obtain the best agreement with the calculated values of the relative luminous efficiency, over the whole range of δ_n . The values of $\log E_n$ for the three subjects obtained in this way are plotted against α in figs. 11 to 13. The value of $\log E_n$ plotted is the mean value obtained from several independent sets of observations (B.H.C. 6, W.S.S. 4, F.W.C. 5). For the subject W.S.S. the observations were made

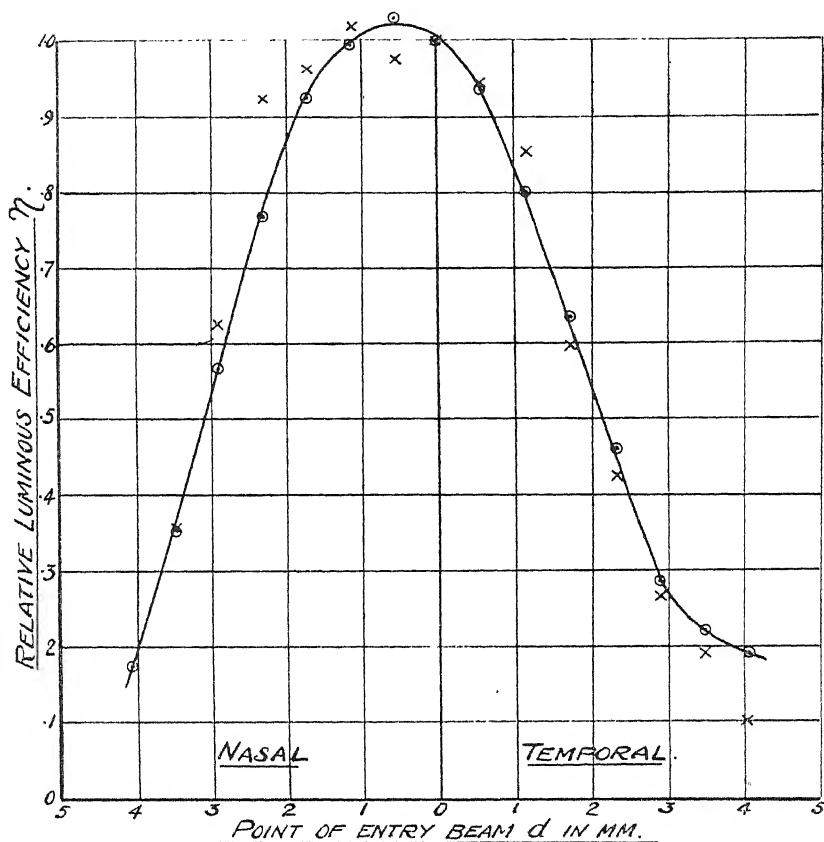


FIG. 10.—Horizontal traverse, subject B.H.C., right eye. \odot Mean of results by flicker method; \times single set of measurements by equality of brightness method.

within a few days of the second series of measurements of the horizontal and vertical traverses, and must be compared therefore with the computed curve based on the later measurements, *i.e.*, with continuous curve II.

The agreement between the calculated curve and the directly observed values of $\log_{10} E$ is satisfactory, although for W.S.S. and F.W.C. there is some evidence of a small systematic difference in shape between the experimental (dotted) and the calculated (continuous) curve. The discrepancy between the directly

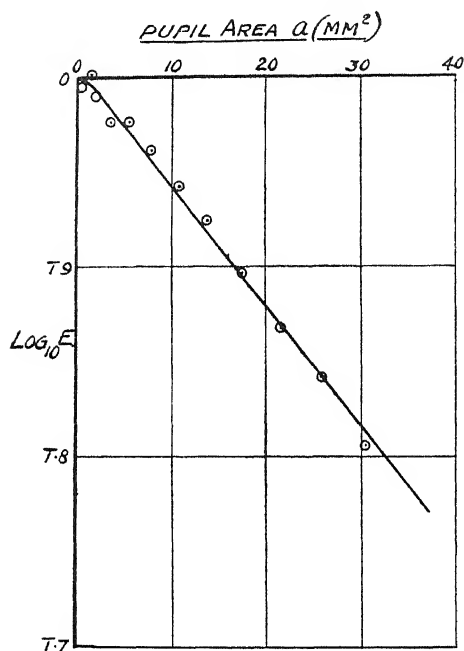


FIG. 11.—Subject B.H.C. Continuous curve; values of $\log_{10} E$ computed from the curves, \odot ; mean values of $\log_{10} E$ by direct measurement.

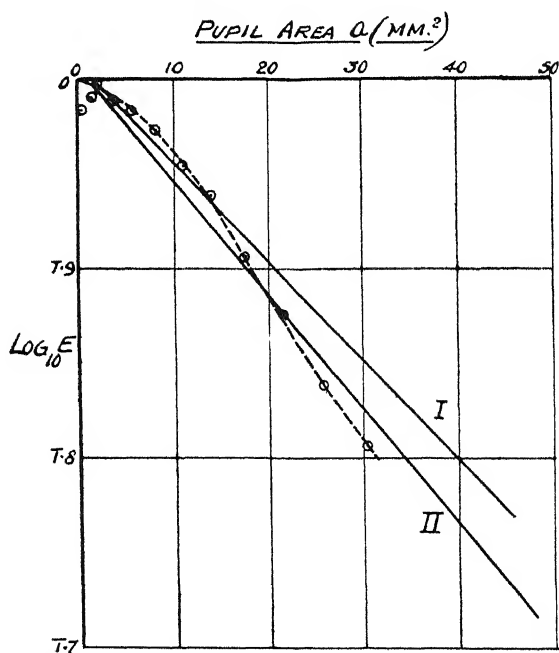


FIG. 12.—Subject W.S.S. Continuous curve I; values of $\log_{10} E$ computed from the η curves—earlier measurements. Continuous curve II; *ibid.* later measurements.

\odot , Dotted curve; mean values of $\log_{10} E$ by direct measurements.

observed (mean) and the computed values of E is at no point greater than 6 per cent. and for subject B.H.C. the maximum discrepancy is 2 per cent. The different sets of observations with the apertures B_n were not, however, in such good agreement as the repeats of the horizontal and vertical traverses. The following points concerning the measurements with the apertures B_n must be mentioned. (1) The brightness of the photometric field varied with the size of the aperture; this should have no effect if the form of the η curve is independent of brightness, or, as we believe, only varies very slightly with

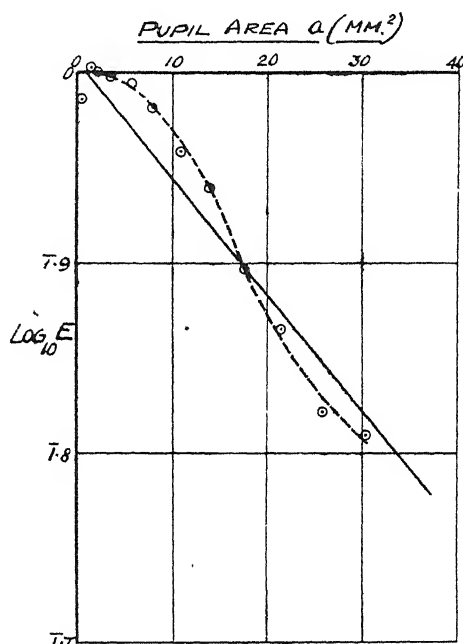


FIG. 13.—Subject F.W.C. Continuous curve; values of $\log_{10} E$ computed from the η curve.

○, Dotted curve; mean values of $\log_{10} E$ by direct measurement.

brightness. (2) Readjustment of the apertures D_1 and D_2 to improve the field appearance obviously could not arise in these measurements, and with the largest apertures there may have occurred some spreading of the field illuminated by J_n , as seen by the subject, owing to the aberrations of the eye. As the maximum diameter of J_n was 6.25 mm. such an effect could not be very marked and in any case it was not noticeable to the subject. (3) Apertures giving images J_n larger than 6.25 mm. were not used because of the interposition of the eyelid. Dilatation of the pupil with a mydriatic was thus unnecessary and in most of these sets of observations no mydriatic was employed.

The measurements of this section go to show that it is legitimate to determine the luminous efficiency of an entrance pupil of any size, shape, or position, by integration of the $\eta(r, \theta)$ curve in the way defined by equation (2). A more detailed study of this point would, however, be useful.

5. *Mode of Origin of the Effect.*

In the scale diagram of an equatorial section of the eye, reproduced in fig. 14, lines are drawn showing the tracks of a ray pencil entering the eye (*a*) through the centre of the pupil opening, (*b*) near the periphery, and terminating on a retinal image P at the fovea centralis. We have to explain why the ray pencil

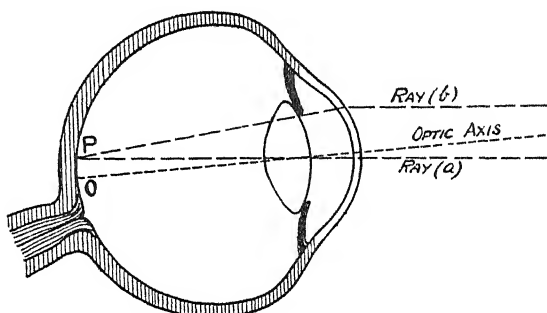


FIG. 14.—Equatorial section of the eye.

incident along track (*b*) is only about one-third as effective in producing the brightness sensation, as the ray pencil incident along track (*a*). There are two possibilities. (1) That the light losses by absorption in the cornea, aqueous, lens and vitreous, or by reflection at the air-corneal, or interior interfaces, are much greater for track (*b*) than for track (*a*); (2) that the (*a*) and (*b*) pencils reach the retina with approximately equal intensities but that the brightness-producing efficiency of rays incident on the retina, varies with their angle of incidence.

Consider first possibility (1). The reflection at the air-corneal interface occurs with an angle of incidence of 0° for pencil (*a*) and an angle of incidence of 31° for pencil (*b*) when the latter enters the pupil at a point 4 mm. from the centre. Assuming the interface behaves as a simple surface separating two optical media having the relative refractive index 1.42 (the value appropriate to the corneal epithelium), the reflections for these two angles of incidence can be computed from Fresnel's laws. The values obtained are 0.030 per cent. and 0.034 per cent. for unpolarized light incident at the angles zero and 31° respectively. The corresponding transmissions equal 1.00 —

$0.030 = 0.970$, and $1.00 - 0.034 = 0.966$. The difference is much too small to play any part in the present effect. Reflections at internal interfaces are between (1/70)th and (1/100)th as intense as the air-corneal reflection and can be dismissed at once.

The absorptions in the refractive media, including the cornea, have been measured by Roggenbau and Wetthauer (1927), for the eyes of the calf and bullock. Their results for the bullock's eye and for light of wave-length $\lambda = 0.58 \mu$, are shown in Table I. Observations on the aqueous are not available for the wave-length 0.58μ , but the results for $\lambda = 0.65 \mu$ show that the absorptions of the aqueous and vitreous are nearly the same and this will be assumed to hold at $\lambda = 0.58 \mu$.

Table I.—Absorptions of Eye Parts of the Bullock (Roggenbau and Wetthauer).

Eye part.	t Thickness in millimetres.	τ Transmission to light of wave-length $\lambda = 0.58 \mu$.	c Absorption coefficient [$\log_{10} \tau = -ct$].
Cornea	1	0.75	0.125
Lens	8	0.83	0.0101
Vitreous	20	0.91	0.00205
Aqueous	4	—	0.00205

Assuming the eye media of man and the bullock to have the same absorptions per millimetre, we can deduce the total absorptions in the ray pencils (a) and (b) in the way shown in Table II. The values obtained refer strictly to

Table II.—Total Transmission and Total Absorption in the Human Eye for Ray Paths (a) and (b). (Deduced.)

	Ray path (a).		Ray path (b).	
	t .	ct .	t .	ct .
Cornea	0.8	0.100	1.2	0.150
Lens	4.3	0.0434	2.8	0.0282
Vitreous	15.9	0.0326	17.2	0.0352
Aqueous	2.5	0.0051	1.7	0.0035
		0.1811		0.2169
$\log_{10} \tau = 1.819$			$\log_{10} \tau = 1.783$	
Total transmission = 0.659			Total transmission = 0.607	
Total absorption = 34.1 per cent.			Total absorption = 39.3 per cent.	

monochromatic light of wave-length $\lambda = 0.58 \mu$, but the effective absorptions for white light, having regard to the visibility curve of the eye which heavily weights the green of the spectrum, will not differ appreciably from the values given. In estimating the path lengths in the different media we have assumed that the lens is accommodated for distant vision and that there is a corneal thickening from the value 0.8 mm. at the centre to the value 1.1 mm. at the point of entry of ray pencil (*b*). It is clear that the difference in total transmission for the two ray paths is insufficient to explain an effect of the order of magnitude of that with which we are concerned. It is perhaps possible that as the cornea thickens in moving away from the centre, its absorption coefficient increases. An increase to about four times its axial value would be necessary for our purpose. We have been unable to find published observations on this point. It seems unlikely that an effect of the magnitude required would have escaped notice.

The above considerations assume the legitimacy of treating the cornea, lens, etc., as ordinary optic media. We know, however, that the cornea and lens exhibit a characteristic structure, and until direct measurements of the light losses in ray paths (*a*) and (*b*) have been made, our conclusion that the observed effect cannot be explained by such losses must be regarded as tentative.

Turning to the second possibility, that the luminous effect of a ray pencil incident on the retina, varies with the angle of incidence, such a "polar sensitivity" of the retina would arise if in one or more of the retina layers there were greater absorption for light passing through obliquely than for light incident normally. At the fovea, the thickness of the retina is about equally divided between the cone layer and the inner layers, the cones being approximately 40μ long and of 2μ maximum diameter. A ray entering the eye at 4 mm. from the axis will pass through the cone layer obliquely (at an angle $\tan^{-1} 4/24$) and will, in fact, traverse on the average about 3 cones ($40 \times \frac{4}{24} = 3.3$). If, to take an extreme case, pigment processes from the pigment epithelium were intruded between the cones so as to enclose them in a kind of absorbing sheath, then the oblique ray would reach one cone only. It is clear that a mechanism of this kind would lead to a reduction of the luminous efficiency for oblique rays of the right order of magnitude. There is, however, as far as we can discover no recorded anatomical evidence of pigment intrusion in the human retina.

We may conclude from the above discussion that the variation of luminous efficiency is probably retinal rather than pre-retinal in origin, but as yet no satisfactory explanation can be advanced.

6. *Practical Consequences.*

In an increasing number of modern photometric instruments, use is made of Maxwellian view and effects of the kind here discussed may lead to appreciable error or to loss of precision if suitable precautions are not taken. The following general observations may be useful in this connection.

For an entrance pupil of given size, shape and position with respect to the pupil of the eye, we can determine a "pupil factor" E which represents the relative luminous efficiency, taking the luminous efficiency for a point entrance pupil at the centre of the eye pupil as unity. In a photometric instrument there are two fields to be compared and where one or both of these are Maxwellian the entrance pupils at the eye for the two fields are not necessarily identical. We can distinguish three cases:—

- (1) If the two entrance pupils are identical then no error can arise (Hartridge, 1919).* The pupil factors E_1 and E_2 are equal and remain equal whatever the position of the eye.
- (2) If the two entrance pupils are not identical in size and position but remain fixed relative to each other, loss of sensitivity and possibly systematic errors may arise. The pupil factors E_1 and E_2 are different and their ratio will vary slightly owing to unavoidable relative motion of the eye pupil with respect to the entrance pupils.
- (3) If the two entrance pupils do not remain in the same relative position for all the measurements, or if the size of one or both of them changes, then definite errors are to be anticipated. Such an arrangement should be avoided wherever possible.

Another direction in which the present effect may play a part is in the correction of visual measurements for pupil diameter. The apparent brightness B_a of an object of external brightness B is usually assumed to be proportional to the pupil area A but, as we have seen, this assumption must be modified and we must write

$$B_a = \text{constant} \times A \times B \times E, \quad (4)$$

where E is the pupil factor. As an example of the use of this equation, let us take König and Brodhun's measurements of the Fechner fraction $\delta B/B$ for different external brightnesses B . These were made with an artificial pupil

* Hartridge in describing a colorimeter in which the beams to be compared are focussed to form images at the position of the eye pupil, states that when the instrument is in correct adjustment these images exactly overlay one another. This is precisely the condition for which no error caused by the present effect can arise.

of fixed area A . In practice we are usually interested in the value of $\delta B/B$ when the eye pupil is allowed to assume its natural size appropriate to the brightness B . To derive this information from König and Brodhun's measurements we note that if B_1 is the external brightness, which gave a given value of $\delta B/B$ in the conditions of König and Brodhun's work, then the same value of $\delta B/B$ will be obtained when viewing a field of brightness B_2 with the pupil at its natural size, provided B_2 satisfies the equation

$$A_1 \times B_1 \times E_1 = A_2 \times B_2 \times E_2, \quad (5)$$

where A_2 is the natural pupil area appropriate to the brightness B_2 , and E_1 and E_2 are the pupil efficiency factors appropriate to pupil areas A_1 and A_2 . Using the data of Prentice Reeves relating pupil area and brightness, and the data of Section 4 for the luminous efficiency of entrance pupils of different areas, equation (5) enables B_2 to be calculated for all values of B_1 throughout the range of measurements. The results of the calculation are shown as a curve of $\delta B/B$ against $\log_{10} B_2$ in fig. 15 (Curve I). Curve II gives the result

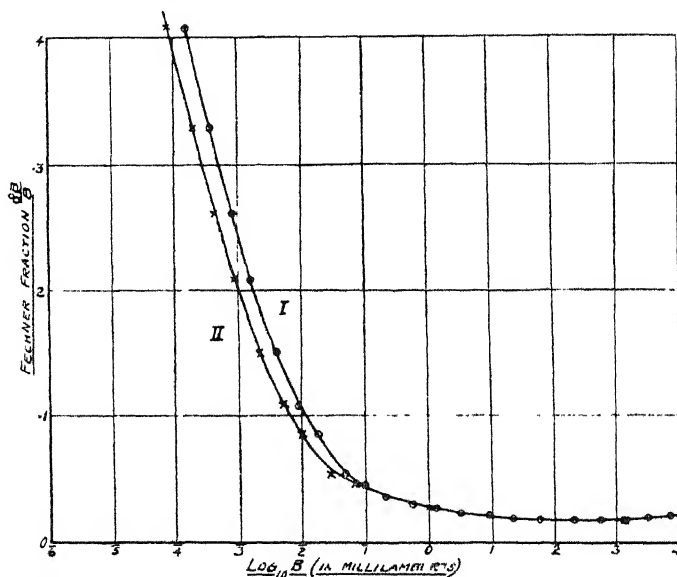


FIG. 15.—König and Brodhun's data for the Fechner fraction $\delta B/B$ reduced for the case when an external brightness B is viewed with the natural pupil.

$$\frac{\delta B}{B} = \frac{\text{Least perceptible brightness difference}}{\text{The greater of the two brightnesses}}$$

Curve I.—Allowance made for the change of pupil area with brightness (Reeves's data for both eyes open), and for variation of pupil efficiency (data from Section 4).

Curve II.—Allowance made for the change of pupil area only.

of a similar calculation in which, however, the pupil factor is assumed to be unity for all pupil sizes.

Addendum.

Since writing the above, a paper by Schroeder (1926) has come to our notice in which the author determines the pupil diameter appropriate to different brightness levels, by an adaptation of Pulfrich's stereo method. In deriving the pupil diameter from his experimental observations, Schroeder implicitly assumes the luminous efficiency of rays entering the eye pupil to be the same at all points. At low brightness levels (corresponding to large pupil diameters) his values for the pupil diameter are considerably below those of Prentice Reeves obtained by photographing the pupil. Schroeder suggests that Reeves's results are open to question, but there can be little doubt that the discrepancy, or in any case a part of the discrepancy, is due to the effect described in the present paper.

A straightforward argument which we need not reproduce here shows that the ratio of Schroeder's value for the pupil diameter to Reeves's value, which, in our opinion, is the true value, should equal the square root of the pupil efficiency factor E appropriate to a circular pupil of diameter equal to the Reeves value. In Table III the fourth column gives the ratio of the Schroeder and Reeves diameters and the fifth column gives the corresponding value of \sqrt{E} derived as a mean from our results in Section 4.

Table III.

Brightness level lux on perfect diffuser of reflection factor unity.	Pupil diameter in millimetres.		Ratio of diameter. Schroeder Reeves	\sqrt{E} From mean results of Section 4.
	Reeves.	Schroeder.		
1000	2.8	3.0	1.07	0.96
200	3.6	3.2	0.89	0.93
100	4.0	3.3	0.82	0.92
10	5.3	3.6	0.68	0.86
1	6.4	3.9	0.61	0.80
0.1	7.2	4.1	0.57	0.76
0.01	7.6	4.3	0.57	0.73

Remembering that we are collating the results of three independent investigations employing different subjects, a comparison of the fourth and fifth columns very definitely suggests that the pupil efficiency factor is responsible for the major part of the discrepancy between Schroeder's and Reeves's results.

The authors have pleasure in expressing their thanks to Mr. F. W. Cuckow who acted as a subject in the investigations.

The work was carried out under the auspices of the Illumination Research Committee of the Department of Scientific and Industrial Research.

Summary.

It is commonly assumed that the apparent brightness of an object is proportional to the pupil area, *i.e.*, that the luminous efficiency of a ray entering the pupil and terminating on the retinal image of the object is independent of the point in the pupil through which it has passed. This assumption is shown to be invalid, and measurements are described which give the relative luminous efficiencies of rays entering the pupil at different points. From these data the overall luminous efficiencies of eye pupils of different diameters are computed, and checked by direct measurement.

The observed variations of luminous efficiency may originate in the eye media or in the retina itself. The evidence at present available is insufficient to decide between these two possibilities. Practical consequences of the effect, in photometry and visual measurements, are indicated.

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Studies on Antagonism.—I. The Effect of the Presence of Salts of Monovalent, Divalent, and Trivalent Kations on the Intake of Calcium and Ammonium Ions by Potato Tuber Tissue.

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Introduction.

The intake of salts by storage tissues has been worked out at some length by Stiles using both the conductivity method and chemical analysis to determine the alteration in concentration of the solution supplied to the tissue. Results of these investigations as well as those of other workers on the subject point to the fact that salts are not taken in as such, but as their constituent ions, which may be absorbed to a very different degree. Stiles (1924) found that the ions were absorbed comparatively rapidly at first, for a period lasting up to 10 hours, after which there was a gradual falling off in the absorption rate so that after 24 hours absorption was only proceeding very slowly.

It was also suggested that the initial rate of absorption depended more on the physical properties of the ions, such as their mobility and the coefficients of diffusion of their salts, and bore, it was found, no relationship to the final position of equilibrium (Stiles, 1919).

In this work we shall be chiefly concerned with the effect of various salts on the intake of the calcium and ammonium ions. That salts do affect one another's absorption by living cells has long been recognized, and to this phenomenon the name "antagonism" has been given. The study of antagonism dates from the time of Loeb and Gies (1902) who first demonstrated its existence. It was shown that the addition of small quantities of either calcium or magnesium chlorides reduced the toxic action of sodium chloride on the eggs of *Fundulus*. It was supposed that the action of the introduced salt was to reduce the amount of sodium chloride absorbed and, as this only took place in the egg stage and could not be demonstrated with the larva of the fish, it was concluded that it was a phenomenon depending on the presence of a membrane.

Szücs (1912) investigated the intake of copper by the roots of *Cucurbita Pepo* and found that in the presence of aluminium salts it was considerably retarded.

From the work of Stoklasa and his colleagues (1922) it became evident that antagonistic action results in a mutual reduction of absorption. They kept plants of *Eriophorum vaginatum*, *Carex riparia*, and *Phragmites communis*, with their roots in complete nutrient solutions, some of which contained aluminium nitrate, some ferric nitrate, and some both these salts. On analysing the experimental solutions after 13 days it was found that less iron and aluminium had been taken in from the solutions containing both salts than from those containing only one.

Antagonism is not only a phenomenon taking place between inorganic salts. Electrolytes are able to reduce the absorption of dyes and the toxicity of poisons. Szücs (1912) found that the toxic action of quinine could be lowered by the addition of various salts. The degree of antagonism was found to be greater with aluminium nitrate than with either calcium or potassium nitrates. He also extended his investigations to the study of antagonism between dyes and electrolytes (1910) and arrived at the conclusion that the antagonistic action of an electrolyte towards a dye depends on the valency and concentration of the kation. He postulated that the intake of the dye is prevented owing to the absorption of the electrolyte by the protoplasm; it would appropriate some of the absorptive surface all of which would be available to the dye in its pure solution, and this, it was held, supported the adsorption theory of salt intake. If we accept this theory, however, the only reason why a salt of high valency has more antagonistic action than one of a lower valency must be that more of it is absorbed. This does not seem to be so, and it will be shown that antagonism cannot be wholly explained on the grounds of mutual hindrance of absorption.

Another theory of the mechanism involved was suggested by Kahho (1921). Using the tissue extension method he investigated the antagonism between salts in their intake by the roots of the yellow lupin as a result of which the "Colloid Precipitation Theory" was advanced. This theory states that the penetrability of any ion varies inversely as its power of coagulating the cell colloids, which power follows the lyotropic series and increases with the valency. This coagulation is supposed to render the outer layers of the protoplasm less permeable to ions so that their entrance is retarded. It would be expected then that monovalent ions should be taken in to a greater extent than divalent which was shown to be the case. The theory also receives support from the work of Hansteen-Cranner (1922) who has observed with the ultra-microscope the precipitating effect of calcium on protoplasm.

The explanation of antagonism that this theory offers is that a divalent ion

will reduce the entrance of a monovalent because there will be more coagulation, that is the cells will be less permeable, than with the latter alone present.

Assuming that Kahho's theory gives a true explanation, any monovalent ion should have comparatively little antagonistic action, because of its relatively small powers of coagulation, and only towards an ion to the left of it in the lyotropic series. Arguing on the same lines, one would expect it to further the absorption of all ions to the right of it in the series. Kahho thought he had evidence of this; from a solution of potassium and lithium chlorides he found "salt" entering faster than from a pure solution of either salt of the same concentration. Owing to the methods used, however, this cannot be taken as definitely proved. The work of Heilbrunn seems to be significant in this respect, so far as it shows that monovalent salts differ from di-, and trivalents, in kind rather than in degree, in their effect on the state of the protoplasm. He showed (Heilbrunn, 1928) that the viscosity of the protoplasm was decreased by calcium and increased by sodium and potassium.

If it can be shown that any salt is capable of furthering the absorption of an ion it will be evident that Szücs's explanation of antagonism is not the correct one. On the other hand, whether one can regard antagonistic action as being solely dependent on the specific reaction of the ions concerned on the protoplasm and entirely neglect the possibility of competition between them for absorption as being an influencing factor, can only be shown by further research.

A review of the literature on antagonism seems to indicate that the majority of previous workers on the subject avoided chemical analysis and exhibited considerable ingenuity in devising other methods of studying salt absorption, methods, the accuracy of which are unfortunately open to criticism, since those not involving direct quantitative determination of the substances used are of only an approximate value at the best. Up to the present the most accurate way of determining the intake of ions is undoubtedly that of chemical analysis, either of the plant or tissue used or of the solution supplied to them. The only objection to the latter method seems to be that the apparent absorption may be due to adsorption on the cell walls. That such adsorption does take place to a certain extent will be shown later, but the absorption cannot by any means be all accounted for in this way.

Most workers have determined the intake of whatever substances they used after a single time-interval. Relatively little work has been done on antagonism following the course of absorption with time. Mann (1924) obtained time-absorption curves of the antagonistic action between dyes and electrolytes. In the majority of experiments, however, he measured the intake of the salts

by determining the anion, but as there is unequal absorption of the ions of a salt this is no criterion of kationic intake and kations are generally regarded as being of primary importance in antagonism.

In this work the course of absorption of calcium and ammonium ions by potato tuber tissue from both pure and mixed solutions is followed by chemical analysis of the solution supplied to the tissue.

Methods.

In the first series of experiments the storage tissue was obtained from King Edward VII potatoes; later great difficulty was experienced in procuring this variety and Kerr's Pink were used. Only results obtained from experiments performed with the same variety are compared.

The method of preparing the tissue was similar to that introduced by Stiles (1924). It was employed in the form of disks 1 mm. thick and 2 cm. in diameter. After cutting, the disks were washed in running tap water for approximately 16 hours and then in four changes of distilled water each of 15 minutes duration. Before being introduced to the experimental solution the disks were lightly dried between filter paper. The bottles containing 80 disks and 200 c.c. of solution were kept in constant agitation in a water bath by means of a trolley, driven backwards and forwards through the water by an electric motor. The temperature of the bath was kept constant, at $25^{\circ}\text{C.} \pm 0.5^{\circ}\text{C.}$, by means of a gas-mercury-toluene thermo-regulator.

Calcium was estimated by the direct volumetric method which with slight modification was found to be reasonably accurate for solutions that had not been in contact with the tissue for more than 10 hours. For longer periods than this it did not give reliable results as the precipitate of calcium oxalate was so finely divided that it would pass through a Whatman 44 filter paper.

For the estimation of ammonium a micro-kjeldahl apparatus was employed with an especially large flask for dealing with large quantities of solution. This method was found to be in all cases most satisfactory.

All solutions were standardized by titrating against decinormal silver nitrate which was also used for chloride determinations. In all titrations micro-burettes were used. Preliminary experiments had indicated that concentrations of 0.02 N were the most satisfactory to use. Accordingly this was the concentration of calcium and ammonium employed, that of the other introduced salts varying from 0.002 N to 0.10 N.

All experiments were conducted at least in triplicate. Chemicals were "A.R.," obtained from British Drug Houses, Ltd.

Results.

The first series of experiments performed was to determine the absorption of calcium and ammonium ions from pure 0.02 N solution of their chlorides. Some typical results obtained are given in Tables I (a) and II (a).

Table I.—Absorption of Calcium Ions.

Progress of the Absorption of Calcium Ions from solutions of 0.02 N CaCl_2 containing antagonistic salts in various concentrations. Absorption of Ca ions expressed as percentage of the original amount present in solution.

(a) CaCl_2 pure—								
Hours minutes	0 1	0 15	0 30	2 0	2 30	4 0	9 0	10 15
Absorption per cent.	1.60	2.30	2.55	2.90	3.00	3.10	3.40	3.40
(b) + 0.02 N NaCl —								
Hours minutes	0 1	0 15	0 35	2 0	2 33	9 0	9 40	
Absorption per cent.	1.4	2.00	2.30	2.40	2.51	2.80	2.80	
(c) + 0.10 N NaCl —								
Hours minutes	0 15	2 0	4 0	7 15	10 0			
Absorption per cent.	0.50	1.55	1.80	1.80	1.80			
(d) + 0.02 N NH_4Cl —								
Hours minutes	0 1	0 15	0 30	2 20	4 12	7 0	10 0	
Absorption per cent.	1.4	1.90	2.35	2.80	2.88	3.00	3.05	
(e) + 0.10 N NH_4Cl —								
Hours minutes	0 30	2 6	4 2	9 15	10 0			
Absorption per cent.	1.0	1.50	1.50	2.05	2.00			
(f) + 0.002 N MgCl_2 —								
Hours minutes	0 1	0 15	3 0	7 45				
Absorption per cent.	1.22	1.80	2.30	2.80				
(g) + 0.02 N MgCl_2 —								
Hours minutes	0 30	1 30	4 9	8 15				
Absorption per cent.	0.76	1.02	1.00	1.11				
(h) + 0.002 N LaCl_3 —								
Hours minutes	2 0	4 0	10 0					
Absorption per cent.	1.0	1.47	2.15					

Considering firstly the data showing the intake of calcium, which are expressed graphically in fig. 1, the small amount entering the tissue after a period of 10 hours immersion is rather striking when the initial, relatively very rapid period of absorption is taken into account. There has entered in the first minute over 50 per cent., and after 15 minutes over 60 per cent. of the total amount absorbed by the disks after 10 hours immersion. Following these initial periods the intake falls off rapidly until after about 2 hours it is only proceeding very slowly. This is well brought out by the graph, fig. 1.

A similar type of absorption curve was found for a 0.04 N solution of calcium chloride. These results for calcium absorption can probably be correlated with those obtained by Miss Redfern (1922). Using the dyes aniline blue and eosin, she found that little of these was absorbed by carrot tissue at equilibrium which was reached so rapidly that the data necessary for the construction of

Table II.—Absorption of Ammonium Ions.

Progress of the Absorption of the Ammonium Ions from solutions of 0.02 N NH_4Cl containing antagonistic salts in various concentrations. Absorption of NH_4 ions expressed as percentage of the original amount present in solution.

(a) NH_4Cl pure—									
Hours minutes	0 1	0 15	1 0	2 0	5 0	8 15	10 0	22 30	24 0
Absorption per cent.	1.75	3.80	6.10	7.70	13.00	17.10	18.00	22.40	22.30
(b) + 0.002 N NaCl —									
Hours minutes	0 15	2 6	5 40	10 36	24 30				
Absorption per cent.	3.50	7.66	13.74	18.40	23.06				
(c) + 0.02 N NaCl —									
Hours minutes	0 15	2 15	5 4	11 0	24 15				
Absorption per cent.	3.50	6.84	12.14	17.55	21.42				
(d) + 0.10 N NaCl —									
Hours minutes	0 15	2 15	10 0	24 0					
Absorption per cent.	2.70	5.90	14.12	16.60					
(e) + 0.002 N CaCl_2 —									
Hours minutes	0 15	2 15	5 9	8 30	24 0				
Absorption per cent.	2.96	6.66	10.45	14.95	18.70				
(j) + 0.02 N CaCl_2 —									
Hours minutes	0 15	2 0	5 0	9 30	24 0				
Absorption per cent.	2.30	4.20	8.20	11.60	13.80				
(g) + 0.10 N CaCl_2 —									
Hours minutes	0 15	2 0	10 0	24 0					
Absorption per cent.	2.30	4.40	8.60	11.30					
(h) + 0.002 N MgCl_2 —									
Hours minutes	0 15	2 40	5 11	9 9	24 20				
Absorption per cent.	2.30	7.82	11.90	16.80	19.60				
(j) + 0.02 N MgCl_2 —									
Hours minutes	0 15	2 37	6 22	9 27	24 0				
Absorption per cent.	2.16	5.70	11.10	12.42	15.90				
(k) + 0.002 N AlCl_3 —									
Hours minutes	0 15	2 36	5 0	9 45	24 0				
Absorption per cent.	2.20	5.56	8.10	11.40	14.20				
(l) + 0.02 N AlCl_3 —									
Hours minutes	0 15	0 30	2 22	5 0	8 34	10 47	23 40	24 0	
Absorption per cent.	1.54	1.74	2.69	3.59	4.69	5.05	0.45	0.78	
(m) + 0.10 N AlCl_3 —									
Hours minutes	0 15	2 0	5 0	9 0	22 10	24 0			
Absorption per cent.	1.4	1.77	2.24	3.00	0.38	0.66			
(n) + 0.02 N LaCl_3 —									
Hours minutes	0 15	2 23	5 12	6 40	9 11	24 0			
Absorption per cent.	1.5	3.10	5.70	5.40	6.73	12.42			
(o) NH_4Cl pure—									
Hours minutes	0 15	3 0	6 0	11 0	24 0				
Absorption per cent.	3.65	8.46	14.10	17.40	19.40				
(p) + 0.02 N NaCl —									
Hours minutes	0 15	3 0	6 0	11 0	24 5				
Absorption per cent.	2.9	7.00	11.30	15.10	16.90				
(q) + 0.10 N NaCl —									
Hours minutes	1 0	3 18	7 45	11 21	24 0				
Absorption per cent.	4.00	8.00	11.40	12.80	13.80				
(r) + 0.02 N KCl —									
Hours minutes	0 15	3 0	7 35	10 40	23 20				
Absorption per cent.	2.7	7.10	12.00	13.42	15.10				
(s) + 0.10 N KCl —									
Hours minutes	1 0	3 18	5 0	7 45	11 21	24 0			
Absorption per cent.	3.6	7.30	8.50	10.50	11.80	12.30			
(t) + 0.02 N LiCl —									
Hours minutes	1 0	3 0	5 19	11 13	24 0				
Absorption per cent.	4.9	14.00	21.00	27.80	29.80				
(u) + 0.10 N LiCl —									
Hours minutes	0 15	1 0	2 45	5 0	10 0	24 0			
Absorption per cent.	3.3	4.60	9.30	15.60	20.40	24.00			

time-absorption curves could not always be obtained. Before suggesting any explanation the absorption of the ammonium ion will be dealt with. A graph derived from the data furnished by Table II may be seen in fig. 3, a consideration of which will show that there is a fairly rapid period of intake lasting up to 10 hours followed by a gradual progression, presumably, to equilibrium. According to Stiles and Kidd (1919) this seems to be the most general course of absorption followed. It is noteworthy that with ammonium there is also an initial relatively rapid period of intake, but after this absorption does not fall off so rapidly as with calcium.

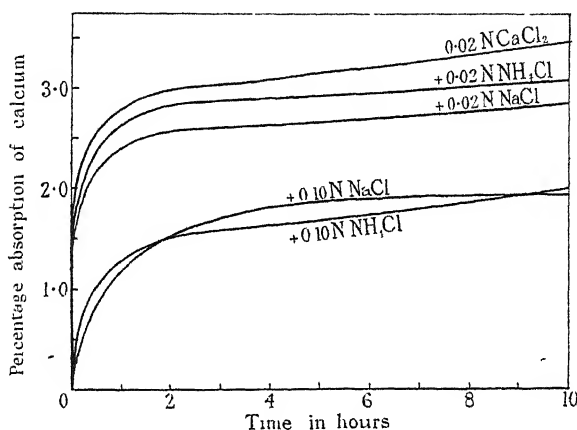


FIG. 1.—Curves illustrating the absorption of calcium ions from a 0.02 N solution of calcium chloride and from solutions containing in addition 0.02 N and 0.10 N sodium and ammonium chlorides respectively.

The similarity of the initial rates of absorption of the calcium and ammonium ions from solutions of their chlorides of the same concentration is very striking when the wide difference in the amounts ultimately entering is considered. The initial intake will be considered, provisionally, as being shown by the determinations performed up to, and including, 15 minutes. If the table is referred to it will be seen that after 1 minute there is 1.6 per cent. and 1.75 per cent. and after 15 minutes 2.3 and 3.8 per cent. of calcium and ammonium absorbed respectively, whereas at 10 hours there is only 3.4 per cent. of calcium taken in compared with 18 per cent. of ammonium. Clearly then there must be different factors deciding the initial absorption from those determining the ultimate position of equilibrium. It has already been mentioned that Stiles and Kidd came to the conclusion that the initial rates of absorption of ions depended more on their physical properties such as their mobility and the coefficients of diffusion of their salts. The above results support this con-

clusion. The ionic mobility of calcium is 51.8 and that of ammonium 64. Accordingly, considering this factor alone, it would be expected that there would be approximately the same amounts initially absorbed from equivalent solutions, with ammonium entering a little more rapidly.

A possible reason for the initial intake of ions depending on these physical factors is forthcoming from a consideration of the nature of the disks of tissue. To the external solution the tissue presents the walls of its cut cells the surface area of which is relatively great. Now these walls are able to adsorb ions as was shown by treating discs, previously immersed in ammonium chloride, with Nessler's solution. A comparatively dense brown precipitate was produced on the outermost cell walls indicating that ammonium was adsorbed there. If then, as seems indicated, initial intake is due to adsorption we should expect it to be dependent on such physical factors as the above. The final amounts entering will, of course, be decided by the specific relationship existing between the ion and the protoplasmic colloidal system of the cells.

The fact that adsorption on the external cell walls does take place will also probably explain the results obtained by Miss Redfern.

The Antagonistic Action between various Salts and the Calcium Ion.

The effect of the presence of monovalent salts on the absorption of the calcium ion was investigated. Sodium and ammonium chlorides were employed in various concentrations and results may be seen in Table I (*b, c, d, e*) and, expressed graphically, in fig. 1. Experiments performed with 0.002 N solutions of these two salts, respectively present with 0.02 N calcium chloride, gave no measurable reduction in calcium intake as compared with that proceeding from the pure solution. From a consideration of the table it will be apparent that there is an appreciable reduction caused by the presence of 0.02 N sodium chloride, namely, in the neighbourhood of 20 per cent. after 10 hours immersion. For the same period the addition of 0.10 N sodium chloride brings about an even greater reduction in the amount of calcium that has entered the tissue.

When 0.02 N ammonium chloride is present there is only a slight diminution in the amount of calcium absorbed. The stronger solution, however, has a similar effect to the same concentration of sodium chloride. It will be evident that the action of these two monovalent salts is of the same order and that the addition of either of them to the calcium chloride solution results in a reduction of absorption of the calcium ion to an extent varying directly as their concentration.

The antagonistic action of the divalent salt magnesium chloride towards the calcium ion will now be considered, it being employed in concentrations of 0.002 N and 0.02 N. It was found impossible to measure calcium accurately in the presence of 0.10 magnesium chloride.

Data obtained are shown in Table I (*f* and *g*) from which the graphs shown in fig. 2 were constructed. A consideration of these will make it evident that magnesium chloride has a much greater antagonistic action towards calcium than either of the monovalent salts used. In a concentration of 0.002 N the absorption of calcium is reduced, on an average, as much as with sodium chloride of ten times the strength and the 0.02 N has much more antagonistic action than either 0.10 N ammonium chloride or sodium chloride.

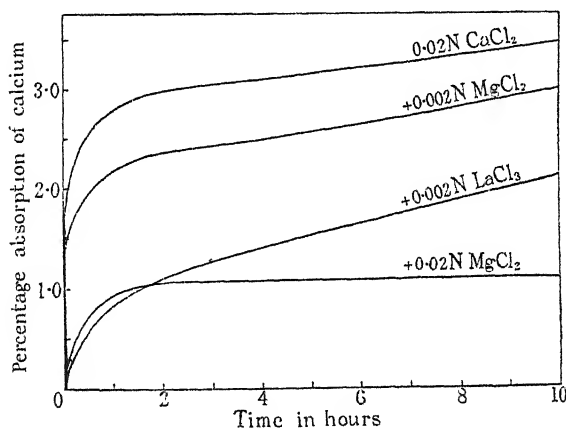


FIG. 2.—Curves illustrating the absorption of calcium from a 0.02 N solution of its chloride and from solutions containing in addition 0.002 N and 0.02 N magnesium chloride and 0.002 N lanthanum chloride respectively.

Similar experiments to the above were conducted with the two trivalent salts aluminium and lanthanum chlorides. Some results obtained with the latter in a concentration of 0.002 N may also be seen in Table I (*h*). A graph drawn from these data is given in fig. 2; it is sufficient to show that lanthanum has a greater antagonistic action than any salt yet used. Solutions containing aluminium chloride in a similar concentration showed no measurable intake of calcium up to 2 hours immersion, but after 7 hours results indicated that amounts varying between 0.80 per cent. and 1.50 per cent. had been absorbed. The extent of calcium intake by the disks from solutions containing 0.02 N calcium chloride and in addition the same concentration of lanthanum and aluminium chloride respectively was so small that it could not be estimated. Calcium is by no means easy to determine in the presence of these trivalent

salts and the data given above must be considered as only approximate, nevertheless they are sufficient to warrant the conclusion that they have a much greater antagonistic action than either mono- or divalent salts. Considered as a whole the results are in agreement with former work on the subject as regards the fact that antagonistic action varies directly as the valency and concentration of the salts employed.

It was decided to confirm the above conclusions by an investigation of the antagonistic action exhibited towards the ammonium ion in its intake by potato tuber tissue. Ammonium is particularly serviceable in this respect for it is taken in very readily by the tissue and there is a very accurate method for its estimation. Results obtained will now be dealt with.

The Antagonistic Action between various Salts and the Ammonium Ion.

The absorption of the ammonium ion from pure 0.02 N solutions of its chloride has already been discussed. There was a very rapid intake in the first 15 minutes owing, it was suggested, to adsorption on the external cell walls of the tissue. Because of this, estimations of ammonium intake were only determined after this initial period and curves, drawn from the data obtained, start from where the first reading was taken and are not produced to the zero point.

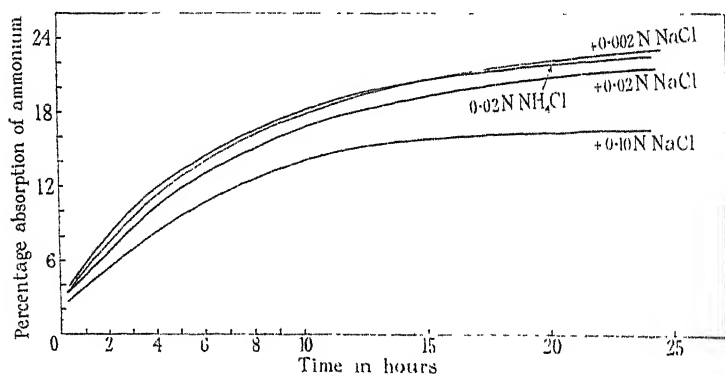


FIG. 3.—Curves illustrating the absorption of ammonium ions from a 0.02 N solution of its chloride and from solutions containing in addition 0.002 N, 0.02 N, and 0.10 N sodium chloride respectively.

The intake of ammonium in the presence of various concentrations of sodium chloride may be seen in Table II (*b, c, d*). Absorption curves constructed from the data furnished by this table are shown in fig. 3 and may be seen

there compared with the absorption from the pure solution. The curve showing ammonium intake in the presence of 0.002 N sodium chloride follows, very closely, the one indicating absorption from the pure solution. There is a slight decrease in the entry of ammonium for the first 6 or 7 hours followed by an apparent increase. It is not proposed to stress this, however, as the differences shown are within the limits of experimental error. Repeat experiments performed with tissue from the same batch indicated that this concentration of sodium chloride has no appreciable effect on ammonium absorption. The addition of 0.02 N sodium chloride always causes a reduction of the ammonium intake; this also applies to the stronger concentration (0.10 N) in the presence of which ammonium intake is reduced by over 28 per cent. after 15 minutes, and at the end of 24 hours by over 25 per cent. The actual course of absorption, up to 24 hours at any rate, does not appear to be affected by the introduction of sodium chloride in any of the concentrations used. The specific amounts taken in are, however, decreased to an extent varying directly as the concentration of the introduced salt. Sodium chloride has then a similar effect on the absorption of both the ammonium and the calcium ion by potato tuber tissue.

Similar experiments to the above were performed using the two divalent salts calcium and magnesium chlorides, data being given in Table II (*e, f, g, h, j*).

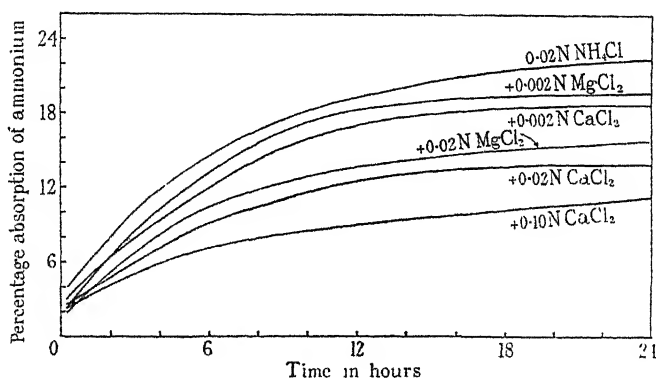


FIG. 4.—Curves illustrating the absorption of ammonium ions from a pure 0.02 N solution of NH_4Cl and solutions containing in addition 0.002 N, 0.02 N and 0.10 N CaCl_2 and 0.002 N and 0.02 N MgCl_2 respectively.

A consideration of the curves derived from these data and shown in fig. 4 will again demonstrate the increased antagonistic action consequent upon an increase in concentration. After 10 hours immersion 0.002 N calcium chloride

decreases ammonium absorption by about 10 per cent., 0·02 N by approximately 32 per cent., and 0·10 N by nearly 50 per cent., as compared with the pure solution of ammonium chloride. There is then a great increase in antagonistic action as compared with sodium chloride of the same concentration. This applies similarly to magnesium chloride which, however, does not have quite such a marked effect as calcium. It may be said to be of the same order and these results agree with those obtained on antagonism exhibited towards the calcium ion as regards the greater antagonistic action of divalent over monovalent salts.

It will be remembered that calcium intake was reduced by the presence of 0·02 N ammonium chloride, and it may now be seen that the absorption of ammonium is reduced in a similar solution ; that is, there is a mutual reduction of absorption.

The two trivalent salts, aluminium and lanthanum chlorides were next used in conjunction with ammonium chloride to determine their antagonism towards

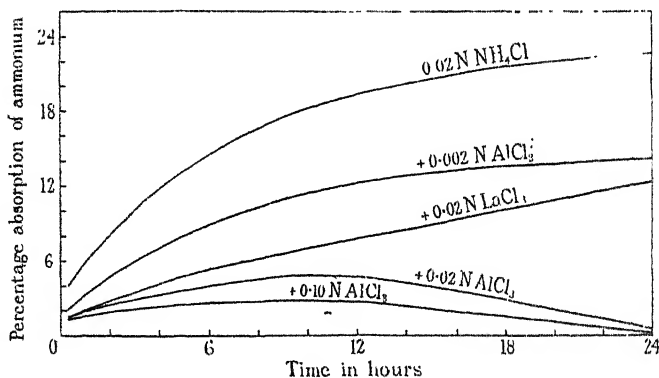


FIG. 5.—Curves illustrating the absorption of ammonium from a pure 0·02 N solution of its chloride and from solutions containing in addition 0·002 N, 0·02 N, and 0·10 N aluminium chloride and 0·02 N lanthanum chloride respectively.

the ammonium ion. The data procured are summarized in Table II (*k*, *l*, *m*, *n*) and expressed graphically in fig. 5. From a glance at the absorption curves the greater antagonistic action exhibited by these salts will be at once apparent. With the weakest concentration of aluminium chloride employed ammonium intake is reduced by over 36 per cent. after a period of 24 hours immersion, this being greater than that caused by sodium, calcium, or magnesium chlorides of the same concentration.

The curves indicating ammonium intake when the stronger concentrations of aluminium chloride are present are quite different from any previously

dealt with. Here absorption is apparently typical up to 10 hours immersion, though, of course, considerably decreased, after which not only does intake cease but the ammonium seems to be returned to the external solution so that after 24 hours less than 1 per cent. remains in the disks. That this type of curve is not general for trivalent salts can be seen from that showing the entry of ammonium when lanthanum chloride has been introduced. Some explanation might be gained from an investigation of the absorption of aluminium by the tissue as well as the effect of the considerable acidity in the solutions containing aluminium chloride. This latter fact may also explain the greater antagonistic action of aluminium as compared with lanthanum.

Activity of Monovalent Salts.

As a result of experiments on salt intake from solutions of potassium and lithium chlorides Kahho (1921) concluded that monovalent salts could further absorption. Potassium and sodium are very similar in most of their properties and as the above experiments show that sodium chloride does not increase the intake of either calcium or ammonium any such property most probably belongs to lithium. In order to obtain definite evidence on this point experiments were carried out on the influence of lithium and potassium chlorides on ammonium intake. At the time when this was contemplated it was found impossible to procure good samples of King Edward VII potatoes and it was decided to use potatoes of the variety Kerr's Pink. The absorption of ammonium from a pure 0.02 N solution of its chloride by this variety was determined and may be seen in Table II (o) and fig. 6. On comparing it with

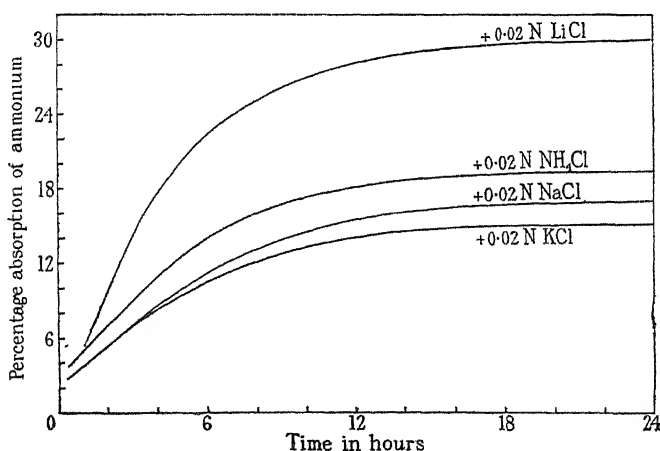


FIG. 6.—Curves showing the absorption of ammonium ions from a pure 0.02 N, solution of NH_4Cl and solutions containing in addition 0.02 N sodium, potassium, and lithium chlorides respectively.

that found for the King Edward potatoes—Table II (*a*) and fig. 3—it will be evident that the absorption curves are very similar but the intake slightly less. In order to have strictly comparable data, as far as possible, for the effect of the monovalent salts, experiments were repeated with sodium chloride. Some typical results are given in Table II (*p, q, r, s, t, u*). It will be apparent from this table and perhaps more clearly from figs. 6 and 7 that with both concentrations of potassium and sodium chlorides employed the former always decreases ammonium intake more than the latter. Lithium is unique in that

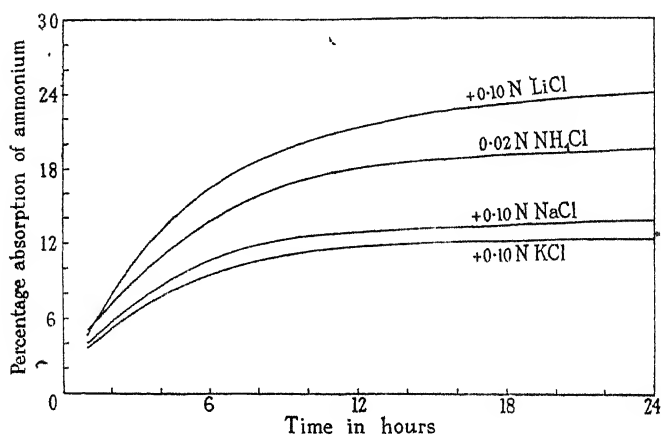


FIG. 7.—Curves showing the absorption of ammonium ions from a pure 0.02 N solution of NH_4Cl and solutions containing in addition 0.10 N sodium, potassium and lithium chlorides respectively.

it actually increases the tissue's intake in both concentrations used but to a greater extent with the 0.02 N present. In these experiments, then, lithium has an opposite effect to potassium and sodium. Another noteworthy feature is that it also causes much more visible exosmosis from the tissue than any of the other salts.

In some experiments conducted in June, 1931, and June, 1932, respectively the weaker concentration of lithium chloride was found to bring about no increase of ammonium intake but a very slight decrease which was, however, not so much as that caused by sodium chloride. It seems significant that these experiments were performed at the same period of the year when the potatoes used would be nearly a year old. This difference in the effect of lithium can, perhaps, be related to seasonal changes in the tissue. As contrasted with what is considered its typical action it was also noticed that there was here no more visible exosmosis from the tissue than was brought about by the other salts.

A point that is here thought worthy of note is that one substance, other than lithium chloride, viz., sucrose, was found to cause an increase in ammonium absorption; this may be seen from the results of experiments summarized in Table III.

Table III.—The Effect of the Presence of 0·02 M and 0·10 M Solutions of Sucrose upon the Absorption of the Ions of 0·02 N Ammonium Chloride by Potato Tuber Tissue. (Absorption of Ions in per cent. of original amount present.)

Time.	Ammonium chloride.		Ammonium chloride + Sucrose		
			0·10 M		0·02 M.
	Kation.	Anion.	Kation.	Anion.	Kation.
Hrs. m.					
0 15	3·3	1·4	4·3	1·6	3·9
3 0	7·9	3·2	9·7	3·8	9·5
5 10	12·6	6·0	13·8	6·25	13·1
9 0	15·5	8·3	—	—	—
10 0	—	—	19·3	9·7	18·5
24 0	18·1	—	21·5	—	20·7

The data furnished by this table are expressed graphically in fig. 8 from a consideration of which it will be evident that the increase brought about varies

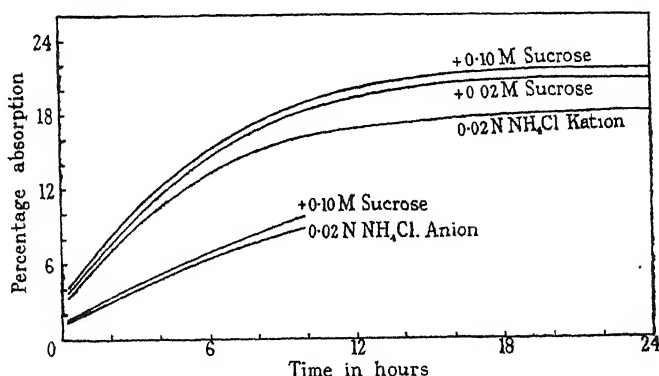


FIG. 8.—Graphs showing the absorption of ions from a pure 0·02 N solution of ammonium chloride and solutions containing in addition 0·02 M and 0·10 M sucrose.

directly as the concentration of sucrose employed. Estimations of chloride showed, it will be seen, that the stronger concentration of sucrose slightly increases its intake also.

From various considerations it does not seem probable that sucrose and lithium increase absorption of ammonium in the same way. As contrasted with lithium the stronger concentration of sucrose causes more absorption than the weaker; moreover, it will now be shown that none of the monovalent salts further the absorption of chloride.

The intake of chloride was determined from 0.04 N solutions of ammonium chloride and from solutions containing 0.02 N ammonium chloride and in addition 0.02 N lithium, sodium, and potassium chlorides, respectively, that is, in all cases the anion concentration was constant at 0.04 N. By determining the chloride intake from the different solutions insight as to the specific effect of the introduction of these kations on anion absorption can be gained. Results are summarized in Table IV from which it will be apparent that sodium and potassium do not influence anion intake to any extent whereas lithium greatly decreases its absorption. This indicates that factors which influence kationic intake do not necessarily have the same effect upon the entrance of anions.

Table IV.—The Absorption of Chloride from a 0.04 N solution of Ammonium Chloride and Solutions containing 0.02 N Ammonium Chloride and, in addition, 0.02 N Lithium, Sodium, and Potassium Chlorides respectively. (Absorption in per cent. of original amount present.)

Time.	NH ₄ Cl.	NH ₄ Cl + NaCl.	NH ₄ Cl + KCl.	NH ₄ Cl + LiCl.
Hrs. m.				
0 15	1.35	1.3	0.8	1.35
3 0	2.45	2.5	2.2	2.25
5 10	3.65	—	—	—
6 0	—	4.35	—	3.55
7 35	—	—	5.00	—
11 36	6.15	6.30	6.00	4.20
24 0	9.00	8.10	8.30	4.45

Discussion.

The work outlined above was undertaken primarily to investigate the nature of antagonism between salts. Before this could be done, however, the absorption of ions from pure solutions of the salts used had to be determined. In doing this results have been obtained which, on the whole, are in agreement with former work on the subject. It has been shown that from 0.02 N solutions of calcium and ammonium chlorides there is a great difference in the amounts of kation absorbed by potato tuber tissue. After 10 hours immersion only

approximately 3.5 per cent. of calcium is taken in compared with 18 per cent. of ammonium. The ions enter comparatively rapidly at first with a gradual falling off until after about 3 hours for calcium, and 10 for ammonium, absorption is only proceeding very slowly. A similarity between the initial rates of absorption of these kations has been detected which, as suggested by Stiles, is probably owing to its dependence more on their physical properties. An explanation of how this may be brought about has been given on p. 458.

The antagonism between various salts and the ammonium and calcium ions, as expressed by the reduction of intake caused by their presence in the same solution as their respective kations, has been determined and as a result the following general conclusions may be drawn. First, the antagonistic action of a salt varies directly as its concentration; secondly, as the results obtained with calcium had indicated, and those with ammonium confirmed, it also depends greatly upon the valency of the salt. In the experiments mono-, di-, and trivalent salts have been used, and at any particular concentration and time it will be evident that antagonistic action varies directly as the valency of the introduced salt. This point is well shown in fig. 9.

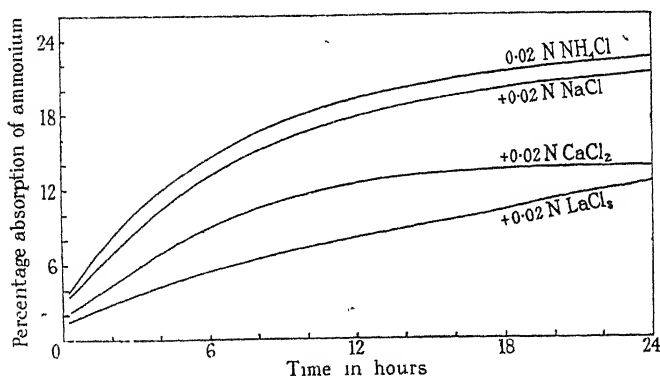


FIG. 9.—Curves illustrating the absorption of ammonium from a pure 0.02 N solution of ammonium chloride and solutions containing in addition 0.02 N sodium, calcium and lanthanum chlorides respectively.

Further dilute solutions of salts have relatively more antagonistic action than stronger ones. Thus with different amounts of calcium chloride present in the ammonium chloride solution after 10 hours immersion; 0.002 N reduces intake 14 per cent., 0.02 N 36.5 per cent., and 0.10 N approximately 50 per cent., so that by increasing the concentration 10 times and 50 times, respectively, antagonistic action is correspondingly increased only by approximately 2.5 and 3.5 times.

Kahho (1921) thought that the effect of monovalent salts was in some cases to further absorption; lithium, it has been shown, definitely has this power, although, in these experiments, this does not seem to apply to sodium and potassium. Now, apart from this work, there seems to be no reason for supposing that the monovalent salts differ from one another, other than in degree, in their effect on plant tissue. According to the work of Heilbrunn (1928) and more recently that of Ingold (1931) monovalent salts have an effect on the plasma-membrane opposite to that of divalent, and trivalent salts. Ingold immersed disks of beetroot, carrot, and potato tissue in solutions of potassium, calcium and lanthanum chlorides. They were then removed, washed, and placed in conductivity water, the exosmosis of electrolytes was then determined and compared with that taking place from tissue previously treated with conductivity water. He found that in all experiments the monovalent salt caused an increase and the divalent and trivalent a decrease of exosmosis as compared with the untreated tissue. He suggests that this is owing to an increase for the monovalent and a decrease by the divalent, and trivalent salts of the permeability of the plasma membrane. Now it will be remembered that when lithium caused an increase in ammonium intake there was an increase in the visible exosmosis from the tissue as compared with the other solutions. This was not so in the experiments with the old potatoes where lithium caused no increase in exosmosis and no increase in ammonium absorption. Thus there seems to be some connection between absorption and exosmosis from the tissue, and it may be concluded with a reasonable degree of certainty that lithium has the same effect on the plasma membrane as potassium and, presumably, sodium, that is, to increase its permeability. The fact that lithium causes an increase in exosmosis of electrolytes was also noticed by Stiles (1919).

Salts that alter permeability would be expected to affect in a similar way the absorption of an ion with which they are enabled to interact. This occurs with divalent, and trivalent salts as regards ammonium intake, but it does not seem to apply to the monovalents. Although all evidence points to the conclusion that they increase permeability, only lithium furthers the absorption of the ammonium ion. Some light is thrown on this matter, however, from a consideration of the effect on absorption of increasing the concentration of the introduced salt. With all salts employed an increase in their concentration brings about an increase in their antagonistic action, that is, a decrease in the intake of ammonium. It is quite understandable how this may be accomplished by the divalent, and trivalent salts; the more there is present the

greater the reduction of permeability. Now the specific effect of lithium is to increase absorption, one would expect therefore, arguing upon the same lines, to get, if anything, a further increase consequent on an increase in its concentration. This was not found; an increase in the concentration of lithium chloride from 0.02 N to 0.10 N decreasing ammonium intake. From this it seems not unreasonable to suppose that an increase in the concentration of one of the introduced salts in itself, and quite apart from its specific reaction on the tissue, causes a reduction in the absorption of the kation of the other salt. This would be expected if competition between the ions for absorption takes place, and it indicates that we cannot entirely neglect Szűcs's theory of mutual hindrance. Whatever the mechanism of salt intake may be it seems very probable that from a solution containing two or more salts there will be competition between the kations for absorption which would be absent in their pure solutions. This competition will tend to reduce their rates of absorption as compared with that taking place from their pure solutions. In our experiments where solutions are used containing two kations it will follow from the above that on an increase in the concentration of one of them there will be a still greater tendency for reduction of intake of the other.

We are now in a better position to consider the results obtained with the various monovalent salts employed. The following explanation is suggested. Let it be supposed that the antagonistic action of one ion towards another depends on two factors; firstly, a tendency for a reduction of absorption caused by competition between the ions, and secondly, the specific effect of the introduced ions on the tissue. With divalent and trivalent ions these two factors will be working in the same direction; both will tend to reduce absorption. On the other hand let us suppose the monovalents are opposed to one another and a balance set up between them. With lithium the balance, as it has been shown, is on the side of a furthering of absorption. Now if the effect of potassium and sodium on the tissue is considered to be much less than lithium, though of the same type, it is not difficult to imagine the balance to be in favour of a reduction of absorption, the first factor entirely masking, even in the more dilute concentrations, any tendency for increasing absorption that these two kations may have. It is hoped to deal with this question in a subsequent paper.

The sucrose experiments also support the suggestion that ionic competition plays a part in antagonistic action. It will be remembered that an increase in the concentration of sucrose brought about an increase in ammonium intake. This is what would be expected. Cells are generally regarded as being

relatively impermeable to sucrose, an increase in its concentration therefore will not bring about a correspondingly great increase in the competition between the ammonium ion and the sucrose molecule for entrance to the tissue. Thus the increased power of sucrose to further ammonium intake in the stronger concentration is not masked by any competitive factor as it has been suggested occurs with the monovalent salts.

In a recent paper Steward (1931) has criticized the value of results obtained on the absorption of solutes by the use of tissue in closed air systems. He says that the respiration of the tissue gradually increases the carbon-dioxide : oxygen ratio which produces an adverse effect on the tissue and causes a gradual cessation of absorption. It is maintained that to be under ideal conditions the tissue should be aerated and have an unlimited supply of oxygen and he has devised a technique to ensure this (1932). In all our experiments a closed air system was used but this, and also the solution, and tissue used was of constant volume. Consequently conditions were, as far as possible, identical and this respiratory factor would be constant. As we were primarily concerned with a comparison of the effect of introduced salts on the absorption of calcium and ammonium ions the conclusions reached are not materially affected.

Unpublished work of the author shows that an increase in the intake of both ions of ammonium chloride can be obtained by the passage of a continuous supply of oxygen through the solution supplied to the tissue. It is noteworthy that Briggs (1930) has advanced a theory, thermodynamically sound, showing how the accumulation of solutes in the cells of *Valonia* and *Nitella* can be explained on the basis of exchange of HCO_3 and H ions for anions and cations, respectively, present in the external solution. This may, perhaps, equally well be applied to potato tuber tissue. Respiration also offers a possible explanation of the effect of sucrose. It is known that immersion in sucrose will cause an increase in the respiration of etiolated leaves and fungi, it may similarly affect the disks of potato and, if Briggs's theory is correct, this would increase the rate of absorption of both ions of ammonium chloride.

In conclusion I wish to thank Professor Stiles for suggesting the problem and for helpful criticism throughout the course of the investigation.

Summary.

(1) In the absorption of the kations of calcium and ammonium chlorides by potato tuber tissue under the experimental conditions employed, there is firstly a relatively rapid period of intake which gradually slows down, so that after about 3 hours, with calcium, and 10 hours, with ammonium, absorption is only proceeding very slowly.

(2) The initial period of absorption of these two kations is very rapid and supports the conclusion that it depends more on the physical properties of the ions. A possible explanation of this is that during this period adsorption is taking place on the external cell walls.

(3) The extent of antagonistic action exhibited towards both the calcium and ammonium ion varies directly as the valency and concentration of the introduced salt.

(4) From solutions containing both calcium and ammonium chlorides there is a mutual reduction of absorption of both kations as compared with that taking place from pure solutions of either.

(5) Dilute solutions of salts have relatively more antagonistic action than stronger solutions.

(6) In concentrations of 0.02 N and over, aluminium chloride has an anomalous effect upon the intake of ammonium. A considerably reduced amount of ammonium is absorbed; this increased gradually for a period of about 9 hours and then apparently returned to the external solution.

(7) Lithium chloride differs from all other salts employed so far as it increases the absorption of ammonium. This may be related to the greater exosmosis from the tissue that it brings about.

(8) Potassium and sodium do not markedly affect the intake of chloride by the tissue; lithium, however, reduces it.

(9) The addition of sucrose causes an increase in the absorption of both ions of ammonium chloride to an extent varying directly as its concentration. This may be owing to its increasing the rate of respiration of the tissue.

(10) It is suggested that the observed "antagonistic" action of an ion depends on the balance existing between its specific effect on the tissue and ionic competition for absorption.

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The Freezing Point of Yolk and White of Egg.

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For the purpose of this note the structure of a hen's egg may be taken to include, in order, a shell, thin white, and thick white enclosing a yolk, which is covered by the thin vitelline membrane.

Under the microscope this membrane is seen to be composed of fibres felted together as in white fibrous connective tissue.

Methods.

Hens' eggs, not more than 24 hours old, were placed in the bath overnight to get into approximate temperature equilibrium. The entire contents of an egg or such portion as was needed for an experiment was then broken into a small metal pail, about 75 c.c. capacity, which was at once covered with a lid lined by wet filter paper to prevent evaporation. The pail was sunk in a bath at a known temperature and left in the earlier experiments for 24 hours and in later experiments 6 hours, before being seeded with ice.

After an interval which varied in different experiments from 2 to 20 days the contents of the pails were searched for signs of ice.

The temperature of the bath was controlled and was placed in a room kept at -1.0° C. by a thermostat. Temperatures were steady to within one-hundredth of a degree Centigrade. The results of the experiments may be summarized as follows :—

(a) At -0.54° C. and -0.56° C.—No ice formed in the yolk when the vitelline membrane was intact, or after it had been pierced by a needle of ice or of glass, or grossly injured by rolling on filter paper to dry the surface.

At -0.58° , -0.60° , and -0.63° C.—No ice formed when the vitelline membrane was intact but in about half the experiments the yolk froze after the membrane had been injured or pierced.

It was observed in these experiments that drying by rolling on filter paper produced general injury so that ice formation in the yolk was scattered over the surface, whereas after puncturing it started only from the injury.

At -0.65° C.—The yolk froze after injury to the membrane but it was doubtful whether ice penetrated the intact membrane.

At -0.68° and -0.70° C.—Ice undoubtedly penetrated the intact membrane.

(b) All the ice in a well-frozen yolk was completely thawed at -0.50° C. and so far as one could see by inspection at -0.56° C.

(c) At none of the temperatures did ice succeed in passing from thin to thick white, instead the latter was dehydrated by water passing across the boundary to the ice column in the thin white. Moran (1926) likewise found ice formed only on the surface of disks of gelatine when cooling was slow.

When seeded separately thin and thick white have the same freezing point, namely, -0.42° C.

Therefore the freezing point of yolk may be taken as -0.57° C., and that of white -0.42° C. Smith and Shepherd (1931) using the Beckmann technique found for eggs 5 days old -0.58° and -0.46° C. respectively.

DETAILS.

Temperature -0.54° C.

(1) An entire egg was broken into each of three pails, and the thin white seeded. After 5, 10 and 20 days the thin white alone was frozen; no ice in the yolk.

(2) The yolk only was placed in each pail and two small areas of its surface dried with filter paper and brushed with a 1 per cent. solution of either nicotine, pilocarpine, or dial (allyl-barbituric acid), or faradized through naked platinum wire. The yolks were then just covered with thin white which was seeded. After 11 days there was no ice in the yolks.

(3) The yolk was floated in a minimum of thin white so as to leave a small circle of the vitelline membrane exposed. After being in the bath for 24 hours the yolk was seeded through the membrane by a fine needle of ice. After 11 days the white was frozen; the yolk unfrozen.

(4) The same as the last, but to avoid any chance of drying, the yolk, after seeding, was covered with a thin plate of ice. After 11 days the white was frozen; the yolk unfrozen.

(5) The same, but the vitelline membrane was pierced with a fine glass needle and then covered with an ice-plate. Result the same.

(6) Moran (1926) found that ice formed only on the outside of a disk of gelatine gel when the rate of cooling was slow, the gel having lost water during the process. To avoid this phase relation each yolk was dried by rolling on

filter paper, and then floated in its pail in liquid paraffin so as to leave a small area uncovered. Twenty-four hours later this area was seeded by being covered with a thin plate of ice. After 11 days there was no freezing.

Controls.—Broken yolks seeded did not freeze at this temperature.

Temperature -0.56°C .

(7) Experiments 4, 5 and 6 repeated with the same result.

Temperature -0.58°C .

(8) Four pails with yolks dried on filter paper, then floated in liquid paraffin and the exposed part covered with a plate of ice. After 18 days two yolks contained ice; two were unfrozen.

(9) Two pails with yolks floated in a minimum of thin white. Yolk was seeded through the membrane with an ice needle and then covered with an ice-plate. After 18 days one yolk had ice-crystals, one was unfrozen.

(10) Two pails as in (9) but the yolk was pierced with a needle of glass. After 18 days both were unfrozen.

Therefore at -0.58°C . such hindrance as there was to ice formation was sometimes overcome and ice-crystals formed within the yolk. It was observed in these and other experiments that rolling on filter paper seriously injured the membranes so that ice formation was scattered over the surface whereas with puncturing it started only from the injury.

Controls.—Broken yolks seeded froze partially at this temperature.

Temperature -0.60°C .

(11) Same procedure as in experiments (8), (9) and (10). After 10 days two, out of the four yolks floated in paraffin, and one, of the four floated in thin white, were frozen.

Temperature -0.63°C .

(12) The entire egg was broken into a pail and seeded in the thin white. After 20 days the thin white was frozen, the thick white and yolk were unfrozen.

(13) The yolk floated in a minimum of thin white which was seeded. After 17 days the white was frozen, the yolk unfrozen.

(14) The same as (13), leaving a small area of yolk exposed which was covered by a plate of ice. After 7 days, in one pail yolk and white were frozen, in the other the white only was frozen.

(15) Various trials with yolks floated in thin white or in paraffin and injured either by rolling on filter paper or by piercing the membrane with a needle of ice or of glass. The small exposed area of yolk was covered with a plate of ice. After 10 days all were well frozen.

Temperature -0.65° , -0.68° , -0.71° C.

(16) The same result as (15), although when the thin white was seeded the ice did not penetrate the thick white.

To sum up. Ice was found for the first time in yolks which had suffered mechanical injury to the vitelline membrane at -0.58° C. and never at -0.56° C. The freezing point of the yolks examined, therefore, lay between these.

A converse experiment was then tried to determine at what temperature ice, already present, disappeared from the interior of the yolk.

Ten intact yolks were placed in pails containing a little liquid paraffin, one in each. After 24 hours each was seeded at -0.60° C. by puncturing and introducing a small crystal of ice. Each yolk was then completely covered with liquid paraffin to prevent evaporation, all the operations being carried out at -0.60° C. Controls with yolks broken into the pails were well frozen next day, but the others were kept for 8 days when each was seen to contain a quantity of ice.

The temperature of the bath was then raised to -0.56° C. for 3 days when all the ice seemed to have gone. To make sure, however, the temperature was raised to -0.50° C. and kept there for 5 days. Careful examination of some of the yolks with a lens failed to reveal ice. To prove its absence the temperature was lowered to -0.63° C. and kept there for 8 days at the end of which time no freezing of the yolks had taken place. The capacity for such overcooling proved the complete absence of ice. Therefore ice could not exist in these yolks at -0.50° and probably not at -0.56° C.

THE FREEZING POINT OF EGG WHITE.

(1) *Temperature* -0.41° C.

In each of four pails the thin white from an egg was placed and in four other pails the thick white. After 24 hours each pail was seeded and the white then covered with liquid paraffin. No ice had formed in any pail after 6 days.

(2) *Temperature* -0.42° C.

The same procedure. After 6 days, 3 out of 4 pails with thin white and 2 out of 4 with thick white contained ice.

(3) *Temperature* -0.45° C.

The same procedure. After 7 days the whites in all the pails were well frozen.

The freezing point of both thin and thick white therefore lies between -0.41° and -0.42° C.

It is important to note that in all the above experiments the time in days at which a certain change is noted does not mean that the change, *e.g.*, freezing, had not taken place earlier: it means simply that for one reason or another it had not been thought advisable or had not been possible to examine the pails earlier.

Note on the foregoing by Sir WILLIAM HARDY, F.R.S.—Recent work on the osmotic pressure of the hen's egg has introduced a sense of uncertainty as to the value of the many comparisons which have been made between osmotic pressures of the blood, body fluids, and surrounding media. The uncertainty pertains not to theory but to a simple matter of fact and, as this involves that most fundamental datum for biological theory—*viz.*, the state of the water in the living cell—there is urgent need to have it cleared up. The fact in dispute is the freezing point of the yolk and white of the bird's egg. Atkins in 1909 by measurements, obviously made with the greatest care, found "no difference between the freezing point of white and yolk of the same egg and a mixture of white and yolk gave the same depression."

Atkins (1909) used the ordinary Beckmann technique and so, too, did Straub (1929) twenty years later, but with a surprisingly different result for he found a constant difference between white and yolk of the hen's egg amounting on the average to -0.15° C. A. V. Hill (1930) confirmed Straub's (1929) finding by a different method. He compared the fall in temperature caused by evaporation with that of water and from the difference calculated the osmotic pressure. Howard (1932) using the Beckmann method again found no difference in the freezing point of white and yolk. In these measurements the yolk was puddled by stirring so that at sometime or another the structure was broken down. Yolk is not only a chemical complex but it is alive, gross mechanical disturbance might, therefore, have the effect it usually has on living cells and cause chemical breakdown with consequent fall of the freezing point. Hale's experiments were designed to explore this possibility by observing directly the freezing point of intact yolk and white.

The difficulties in the way are obvious and not easily overcome. Consider a yolk covered with white with a column of ice, started in the white by seeding, approaching the yolk. When the ice meets the vitelline membrane one of two things may happen: ice may penetrate the pores of the membrane to seed the yolk, or water may move from the yolk to the membrane to condense on the ice face. The second possibility follows from Moran's (1926)

observation when he found that ice formed wholly on the surface of disks of gelatine gel, provided the rate of cooling was slow and that a true reversible equilibrium obtained between gel and ice over a large range of temperature. This must happen if the surface separating ice and gel is plane for, the specific volume of ice being greater than that of water, the formation of ice within the gel would be resisted not only by the positive energy of the new interface, but also by the general cohesion of the gel.

A curious feature about phase lag in the liquid or gel state is the existence of a time limit to overcooling. Why should over-cooling, when once established, not be permanent? The answer, I think, introduces another cause of phase lag, namely, the time taken to orient complex molecules at the new interface. This seems to be the simplest explanation. It takes an hour for a monomolecular film of a fatty acid adsorbed on to a plane solid face to reach equilibrium at ordinary temperature. It is not surprising, therefore, that the orientation needed for the formation of a new interface can take days when the temperature is below the freezing point.

As a minor point Hale's experiments show that the intact vitelline membrane permitted ice to pass to the yolk at -0.65°C . Taking the freezing point of yolk at -0.57°C . it was able to resist no more than -0.08°C . of overcooling. But "intact" yolk must not be taken to mean a yolk with a wholly uninjured membrane for in the act of breaking the yolk into a pail the membrane was obviously violently stretched.

The existence of a difference in the freezing point of yolk and white is fully confirmed and that implies a difference in osmotic pressure, but there is no difference in hydrostatic pressure greater than a delicate, elastic, though possibly tough, membrane such as the vitelline membrane can bear.

The clue to this paradox seems, however, to be simple. It lies in two things: the hindrance to the movement of water in yolk and thick white and the slow leakage of water through the vitelline membrane which, as eggs age, lessens the difference between the *average* osmotic pressure of yolk and that of the white. It is as though there were a number of chokes between a household water tap and the reservoir which together reduced the flow to a trickle. So long as that trickle is unimpeded the pressure difference at the tap is negligible. Try to stop it with the thumb, however, and the whole pressure from the reservoir has to be held back.

I suggest that there is in the egg no sharp change of pressure at either of the surfaces of the vitelline membrane, but there is a gradient from the centre of the yolk outwards of varying, but always slight slope which ends

at a surface whose position depends upon the rate of evaporation from the shell.

Evidence for a hindrance to the movement of water can be found scattered through the paper by Smith and Shepherd (1931), in Moran's (1925) observation that yolk can be readily overcooled—even broken yolk can be held at -7.4° C. and in the various phase lags revealed by Hale's experiments.

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The Blood Circulation of Animals Possessing Chlorocruorin.

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Some of the chemical and physico-chemical characteristics of the respiratory blood pigment chlorocruorin have been dealt with in previous papers (H. M. Fox, 1926, 1932). An experimental investigation is described below of the blood circulation in sabellids and serpulids. These polychæte worms have chlorocruorin in solution in their blood plasma. The work was done in part in the Marine Biological Laboratories of Banyuls, Plymouth, and Tamaris, and my sincere thanks are due to the Directors and staffs of these institutions for their welcome and help.

1. *The Normal Blood Circulation.*

In sabellids and serpulids both the anatomy of the blood system and the mode of blood circulation are peculiar and different from those in other polychæte worms. The best description of the anatomy of the blood system is that of Meyer (1888).

Anatomically the chief peculiarities are these : (1) A considerable proportion of the blood is contained in a peri-intestinal sinus, which replaces a dorsal vessel, except in the most anterior segments ; and (2) the smaller blood vessels, or capillaries, all end blindly (except the capillaries of the œsophageal plexus present in some forms). These blind-ending vessels are found in three situations. (1) There is a single vessel in each primary filament of the crown* with a blind branch into each of the numerous secondary filaments. (2) All capillaries in the body wall branch and end blindly. These can be seen very clearly in the transparent thoracic membrane of the serpulids.† (3) Projecting into each segment of the coelom, and lying freely in the coelomic fluid with its thick suspension of corpuscles or genital products, are numerous unbranched blind-ending capillary vessels.

Physiologically the main peculiarities are the following. Most of the blood vessels in the body are rhythmically contractile. Peristaltic waves force the blood forwards in the peri-intestinal sinus and outwards in the segmental ring vessels which connect the sinus with the ventral vessel. All the blind-ending vessels, in crown, body wall and coelom, are rhythmically contractile. Blood is periodically forced out of these capillaries into the continuous blood vessels by centripetal contractions, to flow back again after a short interval. There is thus a true Galenic circulation in the capillaries. In the case both of the segmental ring vessels and of the capillaries, after each contraction the vessel refills with blood, pauses an instant, swells further to a slight extent, and then again contracts.

The crown consists of two halves, of equal size except in *Spirographis spallanzanii*.‡ At the base of each half there is a single blood vessel. Into each of these basal vessels the single vessels of the separate filaments open, and into the vessel of each filament the blind capillaries of the secondary filaments deverse. Periodically a contraction can be seen to start at the extreme end of each filamentar vessel, passing very rapidly to its base and so expelling most of the blood into the basal vessel. As the contraction passes the base of each secondary filament it is met by the centripetal contraction of the vessel in the latter. The blood is thus forced out of the crown vessels, and it appears

* I refer to the so-called gills as the crown. Morphologically the crown represents the palps (Pruvot, 1885 ; Johansson, 1927). The crown is a ciliary feeding organ (Nicol, 1930), and a respiratory organ (Zoond, 1931).

† *Serpula* can be removed uninjured from its tube by breaking off the posterior end of the latter and blowing through the hole.

‡ In this paper specific names are only given on the first occasion that a species is mentioned.

to flow back again into them under pressure. The contractions are very regularly rhythmic, like those of the continuous vessels in the body and of the blind coelomic capillaries. In a typical case in *Spirographis* at 25° the time from each contraction emptying out the blood to the next contraction was regularly 10·5 seconds, and the time from contraction until the vessel filled with blood again was 6 seconds. The period of contraction, however, of the crown vessels is longer than that of the peri-intestinal sinus. In a typical case of a small individual of *Spirographis*, 5·5 cm. in length, in which the peri-intestinal contractions could be seen through the body wall, these pulsations had a period of 3 seconds at 25° while the vessels in the crown contracted every 11 seconds. The vessels in all the filaments in each half of the crown contract synchronously, but the contractions in the two halves are not simultaneous: one half lags behind the other. In one instance, for example, the vessels of the crown of a *Spirographis* expelled their blood every 12 seconds, but the expulsion in the right half of the crown occurred 4 seconds later than that in the left half.

Experiments involving amputation brought to light a number of interesting facts regarding the rhythm of the vessels in the crown. The terminal halves of filaments were amputated in *Spirographis* and in *Dasychone bombyx*. The cut end of the vessel in the filament closes at once and no blood is shed. In such truncated filaments the blood vessel rhythm is unchanged. The contraction starts from the cut end simultaneously with contractions in the uninjured filaments, and it reaches the base at the same time as these, although it progresses, of course, with half the normal velocity.

If one half of the crown is amputated at its base, contractions of the blood vessels continue in the isolated part for at least 24 hours. Again the cut end of the basal vessel closes at once. The immediate result of the operation is to accelerate the rhythm, although this acceleration is transitory. In a typical case in *Dasychone* at 19° the period of contractions before the operation was 11 seconds. Immediately after amputation the period became 7·5 seconds, in half an hour it was 8 seconds, at the end of 3 hours 8·5 seconds, and next morning the rhythm had returned to 11 seconds, which period it retained all day.

But the most interesting result of amputating half of the crown concerns the simultaneity of contractions in the different filaments. In the intact animal the contractions are synchronous. After amputation, if the cut is made sufficiently far back, the contractions in the filaments continue to be synchronous. But if half of the crown is amputated by cutting through its

base immediately below the point of origin of the filaments, contractions in the latter, while keeping the same period as one another, are no longer synchronous. By trial and error a level for the cut can be found which just permits of the retention of simultaneity in contractions of the vessels of the filaments. This suggests that there is a nerve centre in the base of each half of the crown which controls the contractions. A ganglion has been described in this position (Meyer, 1888), and can be seen in sections.

Finally, a curious result of amputating the crown came to light. Normally all contractions of the vessels of the filaments are centripetal, but after amputation centripetal will often alternate with centrifugal contractions. One or the other direction of contraction will continue for a period of minutes or of hours, changing then to the opposite direction. The phenomenon occurs not only in experiments where the section is made immediately behind the bases of the filaments, but also where the cut is further back, so that the nerve centre is retained which preserves the simultaneity of contractions in the individual filaments. This means that the normal centripetal contractions are controlled not by these nerve centres but by ganglia in the body.

As stated above, the body wall capillaries can be clearly seen in the serpulid thoracic membrane. They can also be observed in the collar and ventral gland shields of sabellids. In the thoracic membrane of each side it can be seen that the capillary system consists in each segment of a basal vessel which sub-divides a number of times to terminate in blind branches. In one such capillary system there is a simultaneous centripetal contraction of all the branches thus expelling the blood, and then the blood flows back again simultaneously into the separate branches. But on examining the living thoracic membrane or gland shields it is apparent that contractions in separate body segments are not simultaneous, nor have they the same period as one another.

The contractions of the blind capillaries projecting freely into the coelom can be watched by opening the body wall laterally in *Spirographis*. The coelom is divided longitudinally into two halves by a dorso-ventral mesentery supporting the intestine. The coelomic capillaries on the side towards the cut in the body wall stop contracting as soon as they are bathed by sea water, but those on the opposite side can be observed through the transparent mesentery contracting in the coelomic fluid. They give the impression of wriggling like so many parasitic worms attached by their heads. They coil up when emptying and then straighten out on refilling. All the capillaries in a given segment contract simultaneously. Their period is approximately, but not always exactly, that of the vessels in the crown. The contractions

may be simultaneous in neighbouring segments, but often the contractions in a given segment are one or more seconds behind those in the segment immediately posterior to it.

The segmental ring vessels have rhythmic centrifugal contractions. When the body cavity of a worm is opened under sea water these vessels stop contracting for a short time, after which they begin to beat at a rapid rate, and then settle down to a constant rhythm with approximately the period of the vessels in the crown. Their contractions are either simultaneous in adjoining segments, or there is a difference of one or more seconds in neighbouring segments. The maintenance of this rhythm between neighbouring segments is independent of the central nervous system, for sectioning the nerve cord between two segments does not alter the respective rhythms of their vessels.

It is not known to what extent this blood system, with its very numerous blind capillaries, is efficient in circulating the blood round the body, as compared with the circulation of other animals which have capillaries connecting arteries to veins. After each expulsion of blood from the blind capillaries by the contraction of their walls it may be that part of the blood returning is the same blood which was expelled. Owing to the absence of corpuscles visible under a low power of the microscope this has not been determined. Yet, owing to the fact that most vessels in the body are contractile, the blind capillaries emptying and refilling, and the trunks circulating the blood by peristalsis, the impression is gained that the circulation round the body is an active one.

2. Reversible Stoppage of the Circulation in Sabellids.

If an individual *Spirographis* is removed from its tube and put, under water, into a glass tube having the same diameter as the natural tube and corked at the posterior end of the worm, the contractions of the blood vessels in the crown can easily be observed through the glass. At 20° these contractions continue at their normal rate for 20 minutes. Then their rhythm slows, and after 30 minutes the pulsations stop completely. If the worm is now taken out of the glass tube, the blood vessel contractions start again immediately, and within 10 minutes they have regained their normal frequency. These experiments were repeated with *Sabella pavonina* with like results. At 15° stoppage occurs in 30–40 minutes, even when the tube is uncorked at both ends, and contractions begin again as soon as the worm is removed from the tube.

These facts indicate that when sabellid worms retire into their tubes and remain there for more than about half an hour their blood circulation stops.

This being so, it is important to know how long the animals can remain in their tubes without injurious effects. Experiments were accordingly undertaken to decide this point. The anterior ends of *Spirographis* tubes were closed by twisting pipe-cleaners around them after the animals had retired into the tubes. The result of these experiments was unexpected. The tube of *Spirographis* is flexible and it is extremely tough except for the anterior end.* In the experiments the anterior ends of the tubes were pulled off until the substance of the tube could no longer be torn apart by the strongest pull with the fingers. The new front end was now closed with a twisted pipe-cleaner. It was found that after some hours each animal had made a new aperture in the side of its tube a short distance behind the pipe-cleaner and had expanded its crown through the hole. In one typical experiment 11 individuals, with tubes between 25 and 30 cm. in length, were operated as just described at 10.30 a.m. At 3 p.m. there was no obvious change in their condition, but at 6.30 p.m. 4 worms had expanded their crowns through newly made lateral holes in the tubes. The holes were about 2 cm. behind the pipe-cleaners. Next morning 3 more had expanded through similar holes, the tubes of 2 others were so fragile 2 cm. behind the pipe-cleaners that they could easily be torn open, while one was dead and 2 were unchanged. By the evening the last 2 had made holes and expanded their crowns. The water temperature was 24°.

It is obvious, thus, that *Spirographis* can remain in its tube unharmed for several hours although its blood circulation is arrested, and moreover that it can pierce the anterior end of a closed tube. In view of the extreme toughness of the tubes and of the fact that before the new hole is made the tube becomes fragile and is easily torn where the hole will appear, a process of external digestion of the mucoid substance of the tube is suggested. The phenomenon is all the more surprising in that, in nature, the anterior ends of the tubes, which project freely into the water, can hardly become accidentally obstructed. Since the holes were made about 2 cm. behind the point of closure of the tubes it would appear that the digestive secretion is produced by the extreme anterior end of the body, at the base of the crown. For although the crowns of the individuals used were between 3 and 4 cm. long, it can be seen by enclosing worms in corked glass tubes that the animals frequently move as far forwards as they can in the closed tubes, crushing the crown to half its normal length in the process. The supposition that the digestive secretion is produced by the

* The mode of formation of the tube with mucus mixed with particles of mud has been described by Nicol (1930).

anterior end of the worm was confirmed by amputating the crowns of 5 individuals and then closing their tubes as before. This was done at 6 p.m. Next morning 3 had made holes in their tubes immediately behind the point of closure, while the remaining 2 tubes were easily torn in a corresponding position.

3. Cause of the Reversible Stoppage.

The most probable reason for the stoppage of blood vessel contractions of sabellids inside their tubes would be either accumulation of carbon dioxide or lack of oxygen. Experiments were accordingly made with sabellids and serpulids to test the effects on the pulsations of blood vessels in various parts of the body of sea water (*a*) containing an excess of carbon dioxide, and (*b*) deficient in oxygen. The result of the experiments, of which typical examples are given in the following paragraphs, was to show that carbon dioxide excess results in a reversible stoppage of blood vessel contractions but that oxygen lack has little or no effect.

When a small *Spirographis** 5.5 cm. in length, through the thin body wall of which the peri-intestinal sinus contractions could be observed, was placed in sea water saturated with carbon dioxide at 25°, the sinus stopped contracting immediately, while the vessels in the filaments stopped after a few minutes. At first there were incomplete contractions in the filaments with the normal period, then the period lengthened and finally contractions stopped, leaving the crown almost empty of blood. Replaced in pure sea water the peri-intestinal sinus contractions recommenced immediately, while the vessels in the filaments resumed their normal rhythm more slowly. In another experiment a *Spirographis* 17 cm. in length, with a normal period of 12 seconds for the contractions of the vessels in the filaments, was placed in carbon dioxide saturated sea water at 23°. After 7 minutes pulsations had ceased in the filaments. Replaced in pure sea water full pulsations restarted suddenly after 5 minutes, with a period of 15 seconds. After 30 minutes the period was 12 seconds once more.

In order to test how long the blood vessel contractions of *Spirographis* can be artificially inhibited while permitting of subsequent recovery, worms were kept for varying times in carbon dioxide saturated sea water. Three cases will be quoted. (1) A worm was kept for 1 hour in carbon dioxide saturated water at 21°, after which it was replaced in pure sea water. At the end of 30

* In all experiments with sabellids and serpulids cited below the worms were out of their tubes unless the contrary is stated.

minutes after replacement there were still no contractions in the vessels of the filaments. Five minutes later feeble contractions started, and 10 minutes after this the pulsations were normal again. (2) Another worm was kept for 4 hours in carbon dioxide saturated water at 21° . Fifty minutes after replacement in pure sea water there were no pulsations yet, but 25 minutes after this the vessels in the filaments were beating with the same rhythm as before the experiment. (3) Twelve hours sojourn, however, in carbon dioxide saturated sea water did not permit of subsequent recovery.

Serpula was next tested. When worms were put into carbon dioxide saturated water at 21° , blood vessel contractions in both crown and thoracic membrane stopped immediately. Replaced 5 minutes later into pure sea water, the contractions recommenced at once.

This inhibition of blood vessel contractions might have been caused not by carbonic acid but by the lack of oxygen consequent on the saturation of the water with carbon dioxide. Experiments were accordingly made with oxygen-free sea water. Nitrogen was first bubbled for 15 minutes through a porous porcelain candle in the water. Then the water was boiled for an hour and cooled under paraffin. The pulsations of the vessels in the crown of *Spirographis*, when animals were kept for 1 hour in this water at 22° , were unchanged. *Serpula* proved to be more sensitive to oxygen lack, but the reaction was in no way comparable with the immediate effect of carbon dioxide. In a typical experiment the period of contractions of the vessels in the crown of a *Serpula* was 14.5 seconds before the experiment, at 24° . At the end of 3, 9 and 34 minutes in oxygen-free water the periods of these contractions had slowed down to 15.5, 18 and 24 seconds respectively. After 9 minutes the thoracic membrane capillaries were still contracting, but these pulsations had ceased after 34 minutes.

Experiments were then made to determine the threshold p_H of sea water containing carbon dioxide for the stoppage of blood vessel contractions. In preliminary experiments with *Spirographis* and *Serpula* it was found that this lies in the region of p_H 6.0. The carbon dioxide "saturated" water used in the previous experiments was Mediterranean water at Banyuls through which carbon dioxide had been passed for 5 minutes in the form of minute bubbles issuing from a porous porcelain candle. The p_H of this water was 5.2. With both *Spirographis* and *Serpula*, water at p_H 6.6 had no effect in 40 minutes on pulsations in the crown, whereas water at p_H 6.0 caused very feeble pulsations in *Spirographis* after 20 minutes, and complete stoppage in *Serpula* after 10 minutes.

More extensive experiments were made with the same end in view on *Sabella* and *Dasychone*. The Plymouth tank water used, after "saturation" with carbon dioxide for 3 minutes in the manner described above, had a p_{H} value of 5.0. For *Sabella* the threshold p_{H} of water containing carbon dioxide for the immediate stoppage of blood vessel contractions in the crown was respectively 6.05 and 6.2 in different individuals. *Dasychone*, however, is better suited to such threshold experiments than the other worms used, for the following reason. When the pulsations of the vessels in the crown of sabellids or serpulids become very feeble it is difficult to determine the end point when contractions have completely ceased. But in *Dasychone* the inner dorsal branch of the palp on either side of the crown* moves slightly with each contraction of the blood vessels in the filaments. This branch thus constitutes a natural lever and the moment when its movement ceases entirely can be accurately observed. This corresponds to the final cessation of contractions in the blood vessels. In this way the p_{H} threshold for the stoppage within 5 minutes of the blood vessel contractions in the crown of *Dasychone* was fixed at 5.95.

From the investigations described above it is clear that the blood vessel contractions in sabellids and serpulids can be stopped reversibly by an accumulation of carbonic acid, and that the threshold p_{H} of such acid water for the immediate or rapid cessation of contractions is about 6.0. It remains to be considered whether this is the cause of the natural stoppage of contractions which takes place when the worms have retired into their tubes.

Individuals of *Spirographis* were placed in glass tubes of the same diameter as their natural tubes. The sea water in the glass tubes was coloured with brom thymol blue and the tubes were corked at both ends. Although the contractions of the vessels stopped in 30 minutes as described above, the colour of the indicator did not alter in that time. It is evident therefore that no appreciable amount of carbonic acid accumulated around the body. Nor can there be a thin stagnant layer of acid water in immediate contact with the body wall, for there are periodical slow movements of the worm's body which must circulate the water around it.

It might be that the closure of the crown, owing to its position in the tube, could cause the stoppage of blood vessel pulsations by a nervous reflex. But this cannot be so, for the following reason. Individuals of *Spirographis* were

* These two processes are outgrowths of the dorsal lip, concerned in the ciliary rejection of faeces and of large particles collected by the filaments (Nicol, 1930). They are Fauvel's "palpes" (1927).

removed from their tubes, and their crowns were held in a closed position by a narrow rubber band placed round the middle of the crown. This did not stop the blood vessel contractions.

It remains probable, then, that the natural stoppage is caused by an accumulation of carbonic acid in the water between the filaments of the closed crown.* This is supported by the following facts. *Sabella* was placed in a glass tube open at each end. After 35 minutes contractions stopped in the crown. With a fine pipette water was then squirted between the filaments. This caused an immediate and temporary recommencement of contractions. Again, in the case of a *Sabella* 7 cm. in length, the contractions in the crown had stopped owing to the worm having been for more than half an hour in an open ended glass tube. When this worm later on moved forwards and projected the ends of its filaments no more than 3 mm. beyond the end of the tube, its blood vessel contractions started again at once.

The next question to be answered was whether the stoppage of blood vessel contractions in water containing carbon dioxide is caused by the increase in hydrogen ion concentration, or by a specific action of carbonic acid. To test the action of hydrogen ions, preliminary experiments were made with *Spirographis* and *Serpula*. By the addition of hydrochloric acid Banyuls sea water was brought to p_H 5.2, the same p_H as that of water "saturated" with carbon dioxide as described above. Two typical experiments using this acid water will be cited. (1) *Spirographis* at 23°: before the experiment contractions of the vessels in the crown occurred once every 10 seconds, and after 11 and 35 minutes in acid water the periods were 11 and 13 seconds respectively. (2) *Serpula*, at 24°: before the experiment the period of contractions in the crown was 14 and in the thoracic membrane 13 seconds, while at the end of 10 minutes in acid water these periods were 21 and 14 seconds. Thus sea water acidified to p_H 5.2 with hydrochloric acid only slightly slows the rate of contractions. This is in striking contrast to the rapid stoppage of contractions brought about by water at the same p_H containing carbonic acid.

Of course, the addition of hydrochloric acid to sea water not only increases the hydrogen ion concentration, but, by reacting with bicarbonate, it also increases the concentration of carbonic acid and dissolved carbon dioxide. It is not certain, therefore, whether the effect of hydrochloric acid just described is due wholly, or at all, to the increase in hydrogen ion concentration of

* More carbon dioxide is probably excreted by the crown than by the body wall, for Zoond (1931) found that the crown of the sabellid *Bispira voluticornis* is responsible for 63 per cent. of the total oxygen intake of the worm when out of its tube.

the sea water. Extensive experiments were undertaken with *Dasychone* to settle this matter. The results of these experiments were briefly as follows.

(1) The Plymouth tank water used had an alkali reserve of 0.0042 N.* The threshold p_H value for the stoppage within 5 minutes of the contractions of the vessels of the crown, when the worms were put into this water after carbon dioxide had been dissolved in it, was found to be 5.95. (2) In tank water acidified with hydrochloric acid the threshold was p_H 5.05. The alkali reserve of this water was changed by the addition of the hydrochloric acid to 0.0002 N. (3) In water, the alkali reserve of which had been raised to 0.0117 N. by the addition of sodium bicarbonate and through which carbon dioxide had been bubbled, the threshold was p_H 6.30.

Now, the carbon dioxide pressures of each of these waters can be calculated from the following equation (Buch, 1930†) :

$$p_H - \log \text{alk} + \log \left(\frac{2K'_2}{C_H} + 1 \right) + \log p_{\text{CO}_2}^{\text{atmos. } 10^{-4}} + \log f - pK'_1 = 4.35,$$

alk is the alkali reserve. $p_{\text{CO}_2}^{\text{atmos. } 10^{-4}}$ is the pressure of dissolved CO_2 in atmospheres $\times 10^{-4}$. f is the volume of CO_2 in cubic centimetres dissolved at the given temperature in 1 litre of sea water in equilibrium with an atmosphere containing 1 part of CO_2 per 10,000. At 15° , the temperature of the experiments, $f = 0.0884$ (C. J. J. Fox, 1909). pK'_1 is the negative logarithm of the first apparent dissociation constant of H_2CO_3 . Its value is 5.91. K'_2 is the second apparent dissociation constant of H_2CO_3 . Below p_H 7, K'_2 has no meaning; the third term therefore disappears from the equation.

The threshold hydrogen ion concentrations (C_H) and the corresponding carbon dioxide pressures (calculated from Buch's equation) for the inhibition of blood vessel contractions in *Dasychone* in the experiments described above are given in Table I.

Table I.

Alkali reserve of water, normality.	Acid added to the water.	Threshold p_H .	Threshold $C_H \times 10^{-7}$.	CO_2 pressure, atmos.
0.0117	Carbonic	6.30	0.5	0.12
0.0042	Carbonic	5.95	1.1	0.10
0.0002	Hydrochloric	5.05	8.9	0.04

* This high value is due to the artificial addition of quicklime to the water in the aquarium circulation (Atkins, 1931; Cooper, 1932).

† I am indebted to Mr. H. W. Harvey for bringing this publication to my notice.

Had carbon dioxide alone been responsible for the stoppage of contractions, the figures in the last column would have been identical in the three experiments, but this is not so. Nevertheless, it is clear from the table that carbon dioxide (or undissociated carbonic acid) has a much greater influence than hydrogen ion concentration.

The greater physiological effects of carbonic acid as compared with stronger acids, due probably to a greater permeability of cell membranes to undissociated acid than to hydrogen ions, is already known from the following facts. The effect of perfusion fluid at a given p_{H} on the respiratory centre of the dog is greater at a high than at a low carbon dioxide pressure (Hooker, Wilson and Connett, 1917), and increased respiratory movements can be attained at a p_{H} higher than that of normal blood if sufficient carbon dioxide is present (Scott, 1918). Jacobs (1920, *a*) put tadpoles into solutions of hydrochloric, formic, oxalic, salicylic, acetic, butyric and carbonic acids, all at p_{H} 3.8. In the carbonic acid the tadpoles were motionless in 5 seconds; in the other acids they were still active after half an hour. Again, tadpoles died in approximately the same time in distilled water saturated with carbon dioxide, at p_{H} 3.8, and in M/4 NaHCO_3 saturated with carbon dioxide which has a p_{H} value of 6.9. The effect was not due to the bicarbonate ion, because sodium bicarbonate alone is innocuous. Jacobs (1920, *b*) did further work on the same lines using flowers of *Symphytum peregrinum* which contain a natural indicator pigment. The same colour change in the acid direction was obtained in the flowers by putting them into distilled water saturated with carbon dioxide and into M/2 NaHCO_3 saturated with carbon dioxide, the latter solution having an alkaline reaction of p_{H} 7.4. Haywood (1927) found that Ringer's solution saturated with carbon dioxide, at p_{H} 5.2, reversibly inhibits the response of frog muscle to electrical stimulation, whereas hydrochloric acid at p_{H} 4.4 has no such effect. Finally, Nomura (1932) showed that the critical p_{H} for the stoppage of ciliary movement in the gill of *Pecten* at 20° is 5.5 for carbonic and 3.8 for hydrochloric acid.

The inhibition of blood vessel contractions in sabellids and serpulids by carbonic acid present in the surrounding water is probably due in part to penetration of the acid from the outside into the muscle cells of the blood vessels, and in part to prevention of the exit from the body of carbonic acid produced in metabolism, owing to the back pressure of carbonic acid outside. On these lines the following fact would be explicable. When an animal is put into water containing carbon dioxide at a p_{H} slightly above the threshold value for stoppage of contractions, the blood vessels beat at a slower rate,

and they retain this longer period of beat for an indefinite time. In one instance the normal period of contraction of the crown vessels of a *Dasychone* at 16° was 11 seconds. Put into Plymouth tank water containing carbon dioxide at p_{H} 6.25 the period had lengthened to 19 seconds by the end of 30 minutes. This slower rate of beat was kept up unchanged for the next 4½ hours. Then the animal was replaced in pure sea water. After 2, 4 and 6 minutes respectively from the moment of replacement the periods of contraction were 13.5, 12 and 11 seconds. In the water containing carbon dioxide there was evidently an equilibrium at a new level between the internal and external carbonic acid.

Nevertheless there is another possible explanation of the inhibition of blood vessel contractions. Carbonic acid in the sea water might stimulate receptors and the inhibition of contractions might be caused by a nervous reflex. Cases have been cited above in which the cessation of contractions in water saturated with carbon dioxide and their recommencement in pure sea water were immediate. Even when the amount of carbon dioxide in the sea water is only sufficient to lower the p_{H} just below the threshold value for inhibition of contractions, the effect of this water, and the subsequent recovery of the animals, may still be immediate. Such rapid reactions suggest a nervous reflex. Yet the following experiments indicate that this is not the correct interpretation.

(1) When a piece of the thoracic membrane of *Serpula* is cut off, capillary contractions continue in the isolated piece. If such a piece is put into water saturated with carbon dioxide, contractions cease immediately. Replaced in pure sea water, contractions start again. In one typical experiment after a piece of thoracic membrane had been in carbon dioxide saturated water for 5 minutes, it was put into pure sea water and 3 minutes after this the contractions recommenced vigorously. Thus the isolated thoracic membrane behaves like the whole animal. If the reactions of its capillaries are nervous, it is obvious that no reflex through the central nervous system is concerned, but it must be due to a hitherto undemonstrated nerve net.

(2) The embryos of the slug *Agriolimax agrestis* possess two contractile vesicles which can be observed through the transparent egg shell. When these eggs are put into water saturated with carbon dioxide the rhythmic pulsations of the vesicles cease within 1 minute. Replaced in tap water, the vesicles start again to pulsate normally within 8 minutes. The isolated muscle cells responsible for the contractions can be clearly seen on the walls of the posterior vesicle. It is improbable that these muscle cells have any nerve connections (Bachrach and Cardot, 1923).

(3) It was found that chick embryo hearts respond to carbonic acid in a similar way to the blood vessels of sabellids and serpulids. The experiments were done with 50 to 70 hour embryos at 39°, the egg shells having been opened. The effect of putting these embryos into Tyrode solution saturated with carbon dioxide (p_H 6.1) was that after 2.4 minutes the regular heart beat gave place to very rapid systolic trembling alternating with pauses in diastole. The pauses gradually became longer until in some cases the contractions ceased. This state is reversible when the embryo is replaced in pure Tyrode solution. In one experiment, for example, the heart had been motionless for 15 minutes when the embryo was removed from the carbonic acid. The heart started again 14 minutes later. When such hearts recommence to beat the rate is at first abnormally rapid, and then it gradually resumes the normal rhythm.

There are no nerve connections with the chick's heart until the sixth day. In the experiments just described, therefore, the effect of carbon dioxide was directly on the muscle cells, and the same was probably true for the *Limax* embryo. This, together with the instance of the isolated serpulid thoracic membrane, makes it very probable that the inhibition of the blood vessel contractions of intact serpulids and sabellids in water containing carbonic acid is caused by a direct effect on the blood vessels, not to a nervous reflex.

4. Artificial Reversible Stoppage of Blood Circulation in other Animals.

It is not only in sabellids and serpulids that blood vessel contractions can be reversibly inhibited by carbon dioxide. Cases of a similar nature have already been cited for the heart of the chick and for the contractile vesicles in slug embryos. Further work showed that this is a more widespread phenomenon in the animal kingdom. A short report is given below of reversible stoppage of contractions for the blood vessels of *Nereis*, and for the hearts of crustaceans, a may-fly nymph and an ascidian.

In both *Nereis pelagica* and *N. cultrifera* the threshold p_H of Plymouth tank water containing carbon dioxide for the stoppage within 5 minutes of contractions of the dorsal vessel was found to be 5.75; the inhibition was reversible. The lengths of the worms used were 2–7 cm. for *N. pelagica*, and 5–15 cm. for *N. cultrifera*. The temperature was 14°.

The heart of *Daphnia pulex* was found to stop within a minute in diastole when the animal was irrigated under a compressorium with carbon dioxide saturated Cambridge tap water; the stoppage was reversible. Neither water brought to p_H 5.0 with hydrochloric acid nor water saturated with nitrogen stopped the heart.

Experiments were made with *Artemia salina* in Banyuls sea water at 21°. Water brought to p_H 5.2 with carbon dioxide stopped both swimming and heart beats within 3 minutes. When the animals were replaced in pure sea water, the heart beat and swimming movements started again and returned to their normal rates. Water through which nitrogen had been passed for the same time as the carbon dioxide, in the experiments just quoted, produced no effect. Water brought to p_H 5.2 with hydrochloric acid slowed, but did not stop the heart. The threshold p_H value of water acidified with carbonic acid for stoppage of the heart beat within 3 minutes was found to lie between 5.7 and 6.0. Keeping animals for at least 50 minutes in water saturated with carbon dioxide still permitted of their full subsequent recovery.

Placing nymphs of the may-fly *Chloeon* in water saturated with carbon dioxide caused the immediate stoppage of heart beats. This was completely reversible when the nymphs were replaced in pure water. Water acidified by hydrochloric acid to the same p_H as water saturated with carbonic acid, and water saturated with nitrogen did not stop the heart beats. It was found possible to keep nymphs in water saturated with carbon dioxide for three-quarters of an hour with subsequent full recovery in pure water, but 1 hour in the acid water was fatal.

The heart beat of the ascidian *Phallusia mamillata* can be observed by shaving the test over the heart. Water saturated with carbonic acid caused reversible stoppage of the heart, while water acidified with hydrochloric acid to the same p_H , and water saturated with nitrogen, did not give this result. The reactions to carbonic acid were sometimes slow, stoppage of the heart taking from 6 to 54 minutes. This variable reaction time depended presumably on the opening or closure of the siphons.

It has been pointed out above that when *Dasychone* is placed in water containing carbonic acid at a p_H slightly above the threshold for the stoppage of blood vessel contractions, the rate of pulsations is slowed, and the slower rhythm is maintained for a long time at a constant rate. This is not true, however, of all animals under similar circumstances. A *Nereis pelagica*, for example, was placed in Plymouth tank water containing carbonic acid at p_H 6.0. Before the experiment the dorsal vessel contracted 16 times per minute. After 6, 15, 17 and 34 minutes in the acid water the rates were 8, 11, 12 and 13 contractions per minute respectively. The dorsal vessel thus adapted itself to the new conditions, tending to regain its former rate of contraction. Again, with a chick embryo the initial rate of heart beat was 130 per minute. After 7, 17, 31, 36 and 41 minutes in Tyrode solution, acidified with

hydrochloric acid to p_H 6.0, the rates were 120, 110, 104, 120 and 120 beats per minute respectively. A similar recovery was observed with *Artemia* in sea water containing carbonic acid at p_H 6.0. This recalls an effect of acetyl choline on the frog's heart found by Clarke (1926). The maximum action of the drug occurred within 1 minute, after which the heart partially recovered.

In conclusion, since in sabellid worms the blood vessel contractions stop when the animals are in their tubes, it was thought possible that a similar phenomenon might occur in molluscs which shut themselves up in their shells. An accumulation of carbon dioxide might stop their heart pulsations. This is not so, however, either in *Mytilus edulis* or in *Halcyon pellucidum*. A small window was made in the shells of a number of individuals of *Mytilus* immediately over the heart. It was found that in such animals, when kept out of water, the heart continued to beat for 12 hours. In *Halcyon* the heart can be seen pulsating through the transparent shell. The heart beat did not stop in animals kept in a damp atmosphere out of water for 8 hours.

5. Summary.

(1) Most blood vessels of sabellids and serpulids are rhythmically contractile, including all the blind-ending capillary vessels in crown, body wall, and coelom. In the continuous vessels the blood is circulated by peristalsis, in the capillaries there is an ebb and flow of the blood.

(2) The rhythmic contractions of the vessels are independent of the central nervous system. The simultaneity of contractions in the vessels of the separate filaments of the crown is controlled by a ganglion at the base of each half of the crown.

(3) After sabellid worms have retired into their tubes for over half an hour, the contractions of their blood vessels cease. The contractions recommence as soon as even the tip of the crown projects beyond the end of the tube.

(4) In spite of the cessation of blood circulation, *Spirographis* can remain uninjured for 8 hours in its tube when the latter is artificially closed. After 8 hours the animal makes a new anterior lateral opening in the closed tube and re-expands its crown.

(5) When sabellids or serpulids, removed from their tubes, are put into sea water the p_H of which has been brought below 6.0 by the addition of carbon dioxide, the contractions of their blood vessels stop. Neither water acidified with hydrochloric acid to the same p_H as the water containing carbon dioxide, nor water lacking dissolved oxygen, produce this effect. The inhibition of

blood vessel contractions due to carbon dioxide is reversible when the animals are replaced in pure sea water.

(6) Carbon dioxide does not inhibit the blood vessel contractions through a nervous reflex. It causes an internal accumulation of carbon dioxide which acts directly on the blood vessel walls.

(7) It is probable that the cessation of blood vessel contractions when sabelids have retired into their tubes is due to the accumulation of carbon dioxide in the water between the filaments of the crown.

(8) The threshold p_H for the inhibition of blood vessel contractions of *Dasychone* was determined in (a) sea water containing dissolved carbon dioxide, (b) sea water of abnormally high alkali reserve containing carbon dioxide, and (c) sea water acidified with hydrochloric acid. From these data it was concluded that both carbon dioxide and hydrogen ions are concerned in the inhibition, but that the former is more important than the latter.

(9) Carbon dioxide also causes the reversible inhibition of contractions in the following cases: (a) the dorsal blood vessel of *Nereis*; (b) the hearts of *Daphnia*, *Artemia*, *Chloeon* nymphs, *Phallusia*, and chick embryos; (c) the contractile vesicles of *Limax* embryos.

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*The Absorption and Excretion of Water by the Mammal. Part 1.-
The Relation between Absorption of Water and its Excretion by
the Innervated and Denervated Kidney.*

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[PLATE 21.]

I. *Introduction.*

When a large volume of water is given by mouth to the normal dog, there is usually a delay of about 10 minutes before the kidney begins to respond, and of about 50 minutes before the response reaches its maximum intensity, fig. 1. It was with the main object of apportioning this delay between the processes involved in the transport of the water, and of determining the relationship between the water-load of the body and the rate of excretion of water by

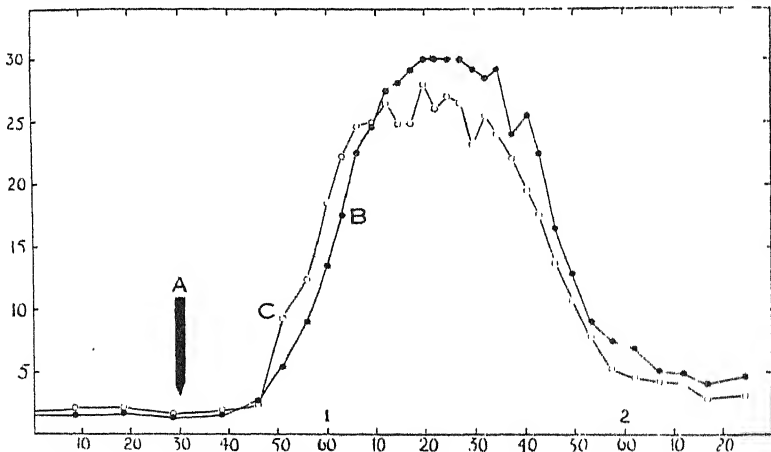


FIG. 1.—Response of right and left kidneys to the introduction of water into the stomach. Ureters extended to the exterior by the method described in the text. At A 250 c.c. tap water at a temperature of 30° C. were given by stomach-tube. B and C are the rates of collection of urine from the right and left kidney respectively.

Ordinate = rate of flow of urine in cubic centimetres per 15 minutes.

Abscissa = time in minutes and hours.

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† Working with a grant from the Medical Research Council.

the kidneys, that the work described in this paper was undertaken. As will be seen later, we have used a method which, in allowing the secretion of one kidney to be collected separately from that of the other, has offered the means of answering cognate questions involving the influence of some factors of physiological significance on the response of the kidney to water-ingestion. To these questions consideration will be given in Part II, where all the facts in their theoretical interrelationship will be discussed.

The delay referred to above, might be allocated theoretically to one or more of several sites and functions. First, the water might be retained in the stomach, its passage into the small gut being accurately and rapidly reflected in its excretion by the kidney. Second, absorption itself might be the principal seat of delay. Third, there might be a latent period between absorption and excretion, the spatial representation of the events occurring in this time not being, of necessity, restricted to the kidneys.

II. *Data in previous Work relating to the Absorption of Water.*

We believe previous work to have demonstrated that the passage of water from stomach to duodenum is too rapid for any appreciable part of the delay between ingestion and excretion to be attributed, with reason, to this phase in the transport of the water. Hirsch (1892), Gley and Rondeau (1893) and Moritz (1901), working on conscious dogs with duodenal fistulæ, all agree that water given or taken by mouth passes immediately through the pylorus. The rapid passage of water from stomach to duodenum has also been demonstrated with almost equal directness by means of X-ray skiascopy and photography (Roux and Balthazard, 1898). Further, our own observations to the effect that when dogs are killed before absorption of water is complete, a large proportion of the residual fluid is found in the small intestine, and not confined to its upper reaches, support this earlier work, and the conclusion is, we believe, warrantable, that the delayed response of the kidney is not conditioned in the normal animal by the rate of passage of water through the pylorus.

Although there are indications in the accounts of past work on the absorption of water that the process is a rapid one, no accurate data are available from which the course of absorption in the normal animal can be inferred with confidence. This being so, it has not been possible to relate the variations with time in the water-load of the tissues, to the rate of water-excretion by the kidneys. The indications referred to are available in three main sources, namely, investigations on (a) the composition of the blood following water-ingestion, (b) the gaseous metabolism of the gut and of the animal following the

introduction or ingestion of water, and (c) the rate of absorption of liquid from intestinal loops.

With respect to the first source, a study of the work of Chiarolanza (1908), Blix (1916), Motzfeldt (1917) and Smirk (1932) in the rabbit, of Rioch (1930) in the dog, and of Priestley (1916, 1921), Rioch (1927) and Govaerts and Cambier (1930, *a, b*) in man, suggests to us that absorption of water is advancing rapidly at a time when the kidney has not begun to respond. With respect to the second, it seems to us possible, in view of the work of Brodie and Vogt (1909) that the early increases in gaseous metabolism in man following water-ingestion, as recorded by Cannon, Querido, Britton and Bright (1927), by Lublin (1928), and by Grollman (1929), and their common antecedence to the increases in rate of urine-flow, are not unrelated to an early and rapid absorption of water from the small intestine.

The third source from which indications of the rapidity of absorption of water may be gleaned, involves sensible demonstration and measurement, and so gives evidence of more direct and satisfactory a nature. The experiments fall into two groups, in one of which dogs were under the influence of a volatile anæsthetic during the observations, in the other not. Into the former group fall the experiments of Hamburger (1896), of Reid (1900, 1902), and of Brodie and Vogt (1910); into the latter, the experiments of Lannois and Lépine (1883), of Röhmman (1887), and of Rabinowitch (1927). Hamburger's figures show that when 0.97 per cent. solution of NaCl is placed in loops of small intestine, about 50 c.c. of water are absorbed by 30 cm. of gut in 30 minutes, giving a rate of absorption of 0.055 c.c./cm./min. From Reid's figures we can calculate that the mean rate of absorption, in those experiments in which water was placed in the loops of gut, is 0.022 c.c./cm./min. In Reid's second paper, a mean rate of 0.018 c.c./cm./min. is found, the solutions in this second series being 2 per cent. and 4 per cent. glucose.* From a loop of gut in a plethysmograph, Brodie and Vogt give figures which show that 0.016 c.c. water was being absorbed per centimetre of gut per minute. When the liquid placed in the loop was 0.93 per cent. solution of NaCl, the rate of absorption was about the same. The mean of the somewhat divergent figures in the four sets of observations in this group is 0.028 c.c./cm./min. The figures of Lannois and Lépine, who worked with unanæsthetized dogs, show a mean rate of absorption of 0.027 c.c./cm./min. for solutions of peptone,* and of glucose,* placed in

* We have included in our calculations the absorption-rates of fluids from such solutions, as the rates appear to differ little from those of water itself. In any case, they are unlikely to give too high a reading to the final value, and it is against a bias in this direction that we particularly wish to guard.

loops of the small intestine. Röhmman used dogs with Thiry-Vella fistulæ, and the mean rate of absorption, when water was placed in the loops, can be shown to have been 0.020 c.c./cm./min. Rabinowitch's figures show an average rate of absorption of 0.036 c.c./cm./min. when solutions ranging from 0 to 0.9 per cent. NaCl, were placed in Thiry-Vella loops of small gut. The mean of these last three sets of figures is 0.028 c.c./cm./min., a figure identical with that obtained from the available data on the rate of absorption of water from the small gut of the dog under anæsthesia. Now Falck (1873) gives, among other data, the length of the small gut in seven dogs, the weights of which were also known. The mean length per kilogram body-weight is 21.6 cm. In a series of 16 of our own, we obtained the figure 27.8 cm. If we apply the average of these two figures, 24.7 cm., to the mean rate of absorption as given by the published work to which we have referred, we find that the time in which a dog, weighing 10 kilograms, might be expected to absorb 250 c.c. water, distributed evenly along its gut, is 36 minutes.

In referring to the published work on the early changes in the blood, and on metabolism, following water-ingestion, we found them to be suggestive of an early transport of fluid from gut to tissues, and indicative that the response of the kidney is delayed not with respect to absorption only. We now see that the rapidity of this transport receives a certain concreteness from the figure, 36 minutes, which we have derived from published data. Its magnitude, however, in view of the paucity and wide scattering of the figures of which it forms the resultant expression, must be considered as conjectural only, though we shall see later how closely it corresponds with that obtained directly from experiment.

III. *Methods and Results of Experiments.*

(a) *Operative Technique and Methods of Observation.*—Briefly stated, our procedure has been to extend the ureters of young bitches, weighing approximately 10 kilograms, to the exterior, to give, some days later, by stomach tube, 250 c.c. warm water, and so to obtain, from measurements of the responses of the kidneys on several such occasions, a curve representing the mean rates of urine-elimination. At a predetermined time after a further dose of 250 c.c. water, the animal is quickly killed, and the volume of fluid remaining in the stomach and small intestine, measured. This procedure is adopted with several animals, and by killing each at a different time after the giving of the last dose of water, a curve is obtained which expresses the normal course of water-absorption with time. In this curve, and the mean excretion-curve of

all our animals, we have most of the data necessary for the comparison of the course of the water-load of the tissues, with that of the response by the kidney.

The bitch is given 1 mg. of atropine sulphate subcutaneously, anaesthesia induced with chloroform and ether (equal parts), and maintained with open ether. With full aseptic precautions, the abdomen is opened in the midline, and the anterior surface of the left kidney exposed. After the peritoneum just medial to the lower pole of the kidney has been incised, the ureter is picked up with forceps, and the upper 3 cm. carefully cleaned. A silk ligature, passed behind it, is used to fix the silver cannula, A, fig. 2, Plate 21, in position, after this has been inserted through an incision in the wall of the ureter, and pushed so far upwards that its perforated extremity lies wholly within the pelvis. The rubber tube B, to which the cannula is attached, is passed dorsal to the ovarian vessels, to enter the peritoneal cavity below them. The intestines are now retracted to the left, the right kidney exposed, and the procedure already adopted with the ureter of the left kidney, repeated with that of the right. One of the ureters is then seized with artery forceps about 2 cm. from its entry into the bladder, and this length freed from its peritoneal investment. Its wall is incised about 5 mm. above the bladder, and a fine glass cannula, C, having a long length of rubber tubing attached to it, passed distally through the ureter, so that its lower end is free in the bladder. The cannula having been firmly tied in position by a silk thread passed around the ureter at its junction with the bladder, the procedure is repeated with the other ureter. The ends of the long rubber tubes are carried well away from the field of operation, and the contents of the bladder expressed through them. The long tubes are removed from the glass cannulae, and the lower ends of the tubing, already attached above to the silver cannulae, are slipped over the glass, and tied firmly in position, after the rubber tubing has been carefully trimmed to the correct lengths. The urine is now being conducted through artificial conduits from the pelvis of the kidneys to the bladder. The next stage consists in opening the bladder, with a view to extending these conduits through the urethra to the exterior. The apex of the bladder is held in artery forceps, and the utero-vesical pouch, with the parts of the peritoneal cavity immediately lateral and anterior to the bladder, packed with gauze. A sagittal incision, about 3 cm. long, is made through the anterior wall of the bladder, and any residual urine removed by means of swabs. To the end of each of the two glass cannulae seen protruding through the ureteric orifices, is now tied a piece of rubber tubing, DD', 25 cm. long, the lower end of which is attached to a steel knitting needle, 20 cm. long and 2 mm. in diameter in its widest part. The

needles are passed down the urethra, and taken by a second assistant, who exerts gentle traction on them until such short lengths of tubing are left within the bladder, as to ensure directness in the artificial paths through which the urine is now conducted. The second assistant then cuts away the two steel needles by an oblique and a transverse incision, respectively, through the rubber tubes, and so prevents any confusion arising as to the source of the urine flowing from each of the tubes. The bladder wall is closed by an interrupted series of silk sutures, the line of suture swabbed with tincture of iodine, and the surrounding gauze-packing removed. The peritoneal, fascial and muscular layers of the abdominal wall are brought together with silk sutures, and the skin incision closed by means of a series of sutures of silkworm gut. In our first experiments, we were in the habit of bringing the two rubber tubes forward along the anterior abdominal wall, and attaching them to short lengths of silver tubing which were, in their turn, fixed to a silver plate, E, fig. 2, Plate 21. This was held just above the upper border of the sternum by a tape passed around the neck. Latterly, we have dispensed with this method of fixing the ureteric tubes, and now cut them across, one transversely, one obliquely, about 1 cm. from the vulval orifice. Whenever it is desired to collect the urine, this can readily be effected by inserting small glass cannulæ, to which are attached lengths of rubber tubing, into the ends of the ureteric tubes. Soiling of the skin and hair by the urine, in the intervals between the periods of observation, is in this way completely avoided. The animals stand the operation extremely well, and are often walking around their kennels within 2 hours of its end.

Several methods, each involving an incomplete short-circuiting of the ureter, were tried before the one described above was finally adopted; but in all of them, kinking of the ureter, with resultant hydronephrosis, was so liable to occur that no confidence was felt in the results. At first, glass cannulæ were tied into the ureters half-way down their courses, and the cannulæ attached by fine rubber tubes to a pair of silver cannulæ, suitably designed for fixation in the lower part of the abdominal wound, and for conduction of the urine to the exterior. Then the suitability, as a method, of the simple passage of cannulæ from the interior of the bladder into the lower ends of the ureters, into which they were ligated, was tested, and the result found to be equally unsatisfactory. A combination of this method with the tying of cannulæ into the ureters in the mid-part of their course, fixing the cannulæ by sutures to the psoas muscle, and connecting them, by means of fine rubber tubing, with cannulæ passing into the ureters at their entrance into the bladder,

and protruding into the interior of the bladder, as already described, was equally unsuccessful. It was not till we found a means which dispensed with the ureters entirely, and allowed the urine to be conducted wholly through an artificial channel, from the pelvis to the exterior, that complete freedom from obstruction, intermittent or permanent, to the flow of urine, was assured.

The silver tubing, from which the cannulae, A, fig. 2, Plate 21, are made, has an external diameter of 2 mm. and an internal of 1 mm. Each cannula is slightly curved, as shown, and has two shallow grooves turned in its wall, the one 13 mm. from its upper extremity, the other 1 mm. from its lower. The former is for the holding of the ligature which binds the cannula within the ureter, just where this is beginning to expand into the renal pelvis, the latter for the securing of the connection of cannula to rubber tubing. As shown in the figure, the wall of the upper part of the cannula, the part lying free in the pelvis of the kidney, is drilled with several holes, each being 0.9 mm. in diameter, and this ensures that small movements of the cannula will in no wise interfere with the continual patency of the conducting channel. The cannulae are polished, and carefully examined with a lens, so as to make sure that no spicules of metal, which might cause undue injury to the mucosa of the pelvis, remain. The rubber tubing used throughout has external and internal diameters of 2.5 and 1.5 mm. respectively. Fig. 3, Plate 21, is an X-ray photograph of one of our animals.*

The method above described, apart from its suitability as a means of comparing the rates of flow and the compositions of the secretions of the two kidneys, one of which at, before, or after the operation may be subjected to interference with either its nervous or vascular supply, presents for the main problem of this paper two advantages over simple perineotomy. First, observations can be made sooner after the operation, since the wound heals by primary intention; second, and more important, the rate of collection of urine in all probability reflects accurately its rate of secretion. In our experience, when urine is being collected by catheter from bitches which have been previously prepared by perineotomy, steadiness in the rate or acceleration of urine-flow is less well assured than in the preparation we have described. This is doubtless due to the initiation of reflex contraction of the bladder-wall by movements of the urethral catheter (Barrington, 1921).

Observations may be begun 48 hours after the operation, and for the succeeding 10 days the animals are in excellent health. At the end of this time,

* We are indebted to Professor H. A. Harris for this photograph, which was taken in the Department of Anatomy, University College.

or later, the uretero-vesical cannulæ begin to loosen, and the general health of the animal to fail. For a minimum of 10 days, however, observations may be repeatedly made, with the assurance that the responses are those of normal animals. The animals are watered night and morning, given half a pound of meat, with biscuits *ad libitum*, at 6 p.m., and taken for a short run on the roof of the building each day.

When observations on the flow of urine are to be made, the animal is placed in a Pavlov stand, and the apparatus shown in fig. 4, Plate 21, strapped in position. It consists of a metal plate A, shaped to the upper chest between the forelimbs, and held there by tapes passing over the neck and back of the animal. Glass cannulæ, with rubber tubing attached, are passed into the ends of the tubes protruding from the vulva, and connection thus made with the glass tubes C, which turn to discharge into the graduated glass vessels E. These are held by spring candle-clips D, and the central disposition of the tubes C is maintained by short lengths of pressure-tubing, slipped over them and fitted loosely into the mouths of the collecting vessels E.

(b) *The Excretion Curves.*—Our routine has been to put the dog, which has taken nothing by mouth except water since the previous evening, in the stand at 9.30 a.m., and to screen it, so far as possible, from a changing external environment. No one with whose appearance the dog is unfamiliar is allowed to come near it during the observations. These precautions are necessary if the parallelism of repeated responses is to be assured (*Cf.* MacKeith, Pembrey, Spurrell, Warner, and Westlake, 1923). Half an hour later 250 c.c. tap water at a temperature of 30° C. are given by stomach-tube. We chose 250 c.c. as this, with the size of animal on which we were working, while giving a well-marked response, was not sufficiently large to prevent this response from being peaked in nature. The time at which the maximum rate of secretion was attained, could thus be readily determined. The water was given over a period of 2 minutes, and the volumes of urine were recorded every 5 minutes, or more frequently at high rates of flow.

The urine dropped from the cannulæ in a perfectly regular manner. It was only at the high rates of flow that any rhythm was observed in the rate of appearance of the urine. As example we give the following note, made at the time of one of the observations: "Drops of urine fell quite steadily at the lower rates. At high rates, after water-ingestion, the flow became rhythmic, 3 or 4 drops (*i.e.*, about 0.2 c.c.) falling in rapid succession every 4 or 5 seconds." The rhythms of the flows from the two kidneys were independently conditioned, and previous denervation of the kidney was without apparent influence upon them. We believe these pulses to arise in the pelvis of the kidney, and to be the fragmentary expression of that fuller rhythm observable even at low rates of flow, when the ureter preserves its normal function. This condition was apparently present in Kierulf's (1853)

experiments made on dogs, presumably without the use of an anæsthetic. He states: "Die Excretion geschah, wie gewöhnlich, stossweise mit jeder 15ten bis 20sten Sekunde." We would correlate our own observations of the transition of a continuous excretion at low rates, into a discontinuous one at high, with those of Frey (1906), which deal with the increased frequency of ureteric contractions produced by a rise in the intra-ureteric pressure. (*Cf.* Bernard, 1855, pp. 331 and 338.)

On the curves which represent the rates of urine-flow, the time of any plotted point is the mean time of the interval during which the sample of urine, to which this rate refers, is collected.

When the response was complete, 3 to 3½ hours after the beginning of the observations, the animal was allowed to run free in the laboratory for half an hour, then replaced, and, half an hour later, the response to a second dose of water was recorded. In this way a series of responses was obtained, the members of which ran closely parallel to one another. This is illustrated in fig. 5,

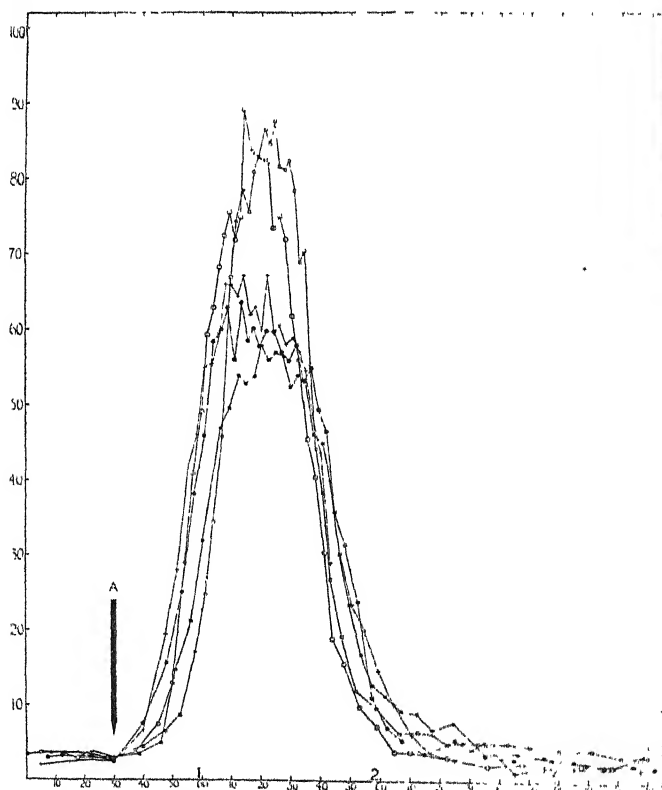


FIG. 5.—Dog No. 46. B, C, D, E and F are the responses of the dog to 250 c.c. doses of water, given during three consecutive days, by stomach-tube. The water was given over a period of 2 minutes at A, and the arrow marks the mid-time of the administration. The time of each plotted point is the mean time of the interval during which the volume of urine was collected. Ordinate = rate of secretion of right and left kidneys in cubic centimetres per 15 minutes. Abscissa = time in minutes and hours.

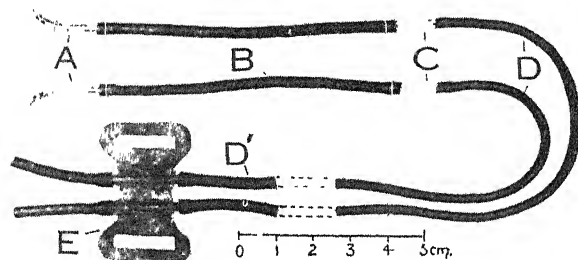


FIG. 2.—Cannulae and tubing used in extending the ureters to the exterior. For description see text.

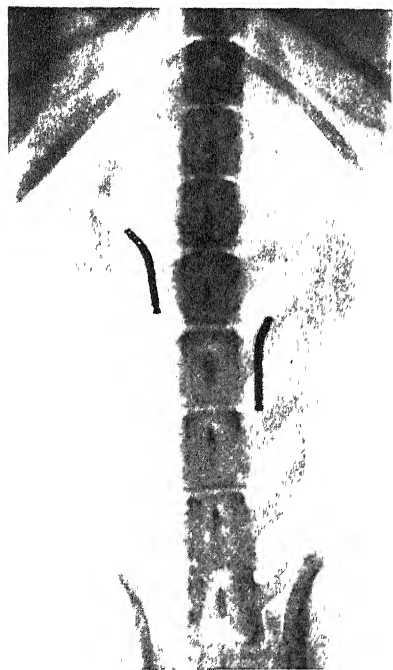


FIG. 3. X-ray photograph of the abdomen of one of the dogs. The photograph was taken post-mortem, and shows the silver cannulae projecting into the pelvis of the kidneys. The perforations in the wall of the upper part of the cannula on the right side of the animal are clearly visible.

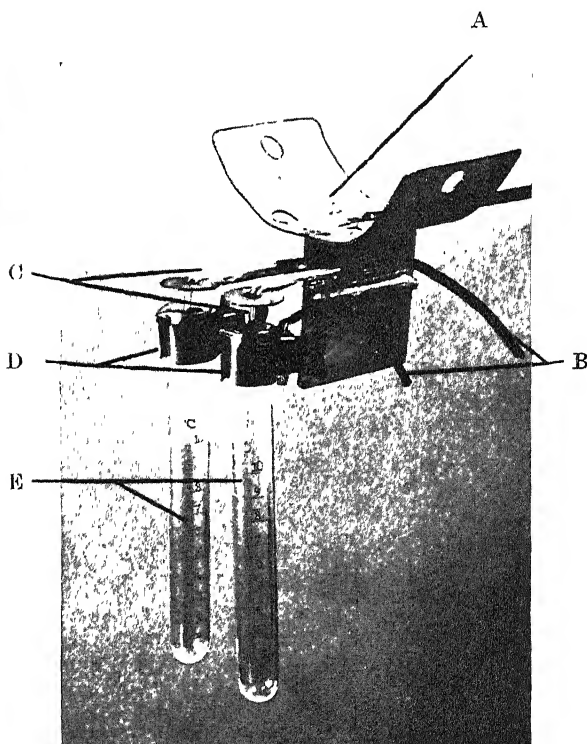


FIG. 4.—Apparatus used for collecting urine during the observations. For description see text.

where five consecutive responses to the introduction of 250 c.c. water into the stomach of one of the dogs, are given. Each curve represents the sum of the rates of secretion of the right and left kidneys, and from these a mean curve is drawn. The data from these observations also serve for the construction of two further series of curves representing the total volumes of urine secreted, and the mean of these plotted against time. The latter is shown in fig. 6. It sometimes happened that the first response obtained in

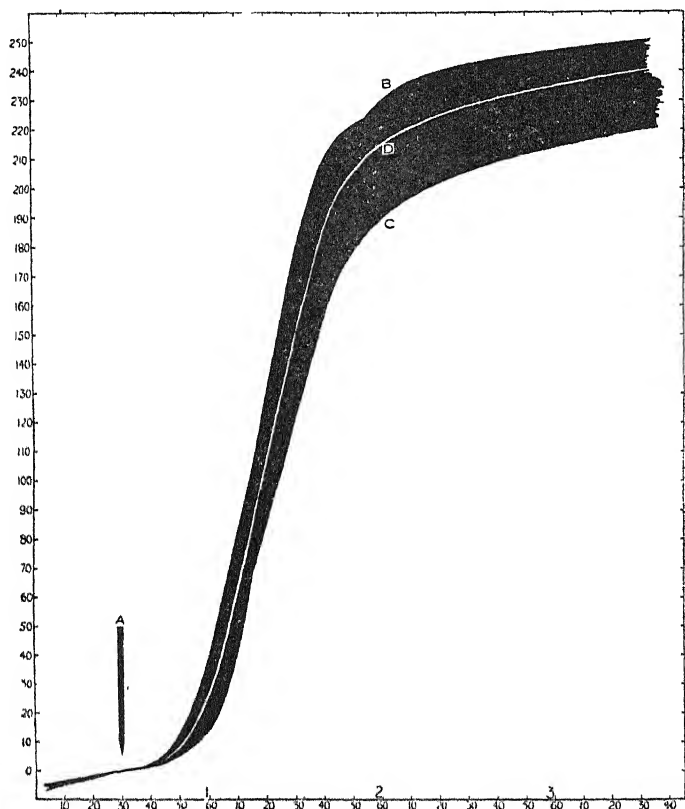


FIG. 6.—Dog No. 46. Total volume of secretion plotted against time. B and C are the extremes and D the mean of the responses. Ordinate = volume of urine secreted in cubic centimetres. A and abscissa as in fig. 5, *q.v.*

the day was small and delayed, and such has been taken to signify that the animal's tissues were not optimally hydrated. Occasionally this response was so small as to be barely appreciable, and we were surprised to find that such a degree of tissue dehydration could accompany the refusal of the animal to drink water, when offered it. One of our animals, for example, having refused the offer of water, was given 250 c.c. by stomach-tube. No definite increase in

the rate of urine-flow was detected during the succeeding 3 hours. It was only after repeating the dose of water, that a diuretic response was obtained, and this, even, was considerably below the responses given by this animal when "optimally" hydrated (*cf.* Ginsberg, 1912, p. 385). Responses which were obviously submaximal have been excluded from the data with which this paper deals.

Twelve dogs, each operated upon as already described, have been subjected to the procedure outlined above, and, in nine of these, the results have been sufficiently satisfactory for mean rate- and total volume-curves to be constructed with confidence. With the remaining three animals, difficulties were encountered. One, although apparently quite fit, persistently vomited the water which had been introduced. Another panted to such a degree, that 153 c.c. only were excreted by the kidneys in the $2\frac{1}{2}$ hours following the giving of the water. This poor response was probably due to the concomitant loss of water by vaporization, as it followed one of 108 c.c. which had terminated only an hour before the giving of this second dose of water. On each occasion the panting appeared to be conditioned by the giving of the water as it began 10 minutes later. The phenomenon was seen only occasionally during the course of this work. Often, however, the dogs' muzzles were observed to become more moist 4 to 15 minutes after the giving of the water. The third dog showed an abnormally protracted response, and, post-mortem, was found to have an extensive ulcerative enteritis.

The number of observations on each of the nine dogs varied between two and five, and since in the construction of the absorption-curve, to be described in a moment, one point only was obtainable from each animal, the means of the mean rate- and mean total volume-curves for each animal were constructed, rather than the means of all corresponding curves. In this way any undue weighting of the means towards the curves obtained from the animals on which the largest number of observations had been made, was avoided. The curves are shown in figs. 7 and 8, FBGIII, fig. 7, representing the mean of the nine mean rate-curves, and EAFG, fig. 8, that of the nine mean volume-curves.

(c) *The Absorption Curve.*—After receiving a further dose of 250 c.c. water, the dog is rapidly rendered unconscious with chloroform, at a predetermined time, and the heart immediately punctured through the chest wall. The time between the beginning of the administration of chloroform and the puncture of the heart was 2 to $2\frac{1}{2}$ minutes. After rapid opening of the abdomen, the portal vessels are clamped, the cardiac orifice ligated, and the stomach and

intestines excised. They are opened over a dish, the contents and scrapings from the mucosa strained through fine muslin, and the volume of the filtrate measured. In this way the volume of water absorbed in a given time is obtained.* The results of ten observations of this nature are shown as white

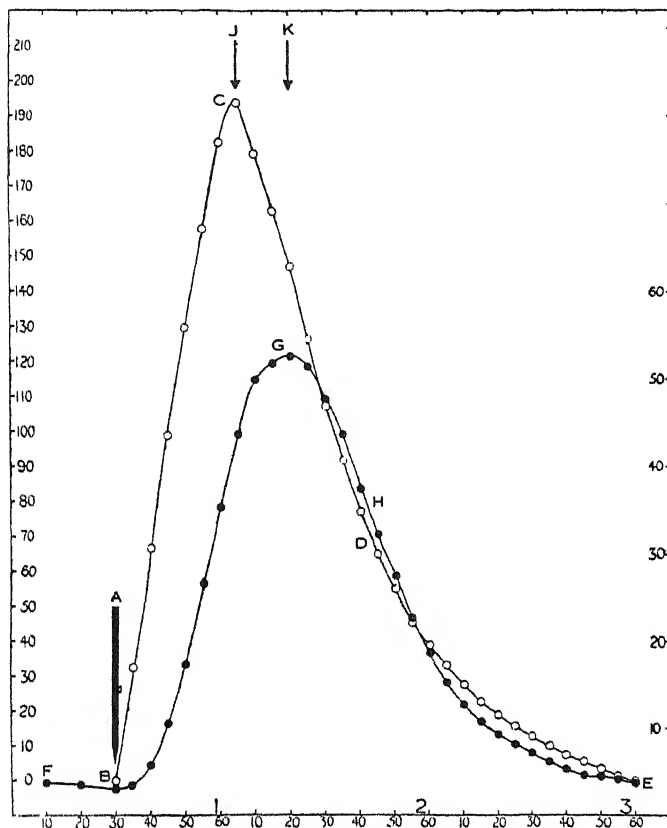


FIG. 7.—F, B, G, H, E = the mean of the mean rates of secretion obtained from nine animals, Nos. 46, 48, 52, 53, 54, 55, 58, 63, and 65. Its ordinate, in cubic centimetres per 15 minutes is scaled on the right side. B, C, D, E represents changes in the water-load of the tissues with time. The ordinate of this curve is in cubic centimetres and is scaled on the left side. A = time of giving the water. Abscissa = time in minutes and hours. The interval JK between the peak of the water-load curve and the maximum rate of urinary secretion is 15 minutes.

circles on the black area of fig. 8, and the time-element in each is taken as being the interval between the mid-time of introduction of the water, and the time of puncture of the heart. The white line AKD is the mean of lines drawn

* Pieces of the kidneys of these animals were fixed in formalin immediately post-mortem. An examination of specimens stained with hæmatoxylin and eosin revealed no abnormality.

from A through each of these points, and will be referred to as the absorption-curve. Two points, I and J, lie outside the black area. The former was obtained from the dog whose gut, as mentioned above, was the seat of extensive inflammatory and ulcerative changes. It seems to us not unreasonable to

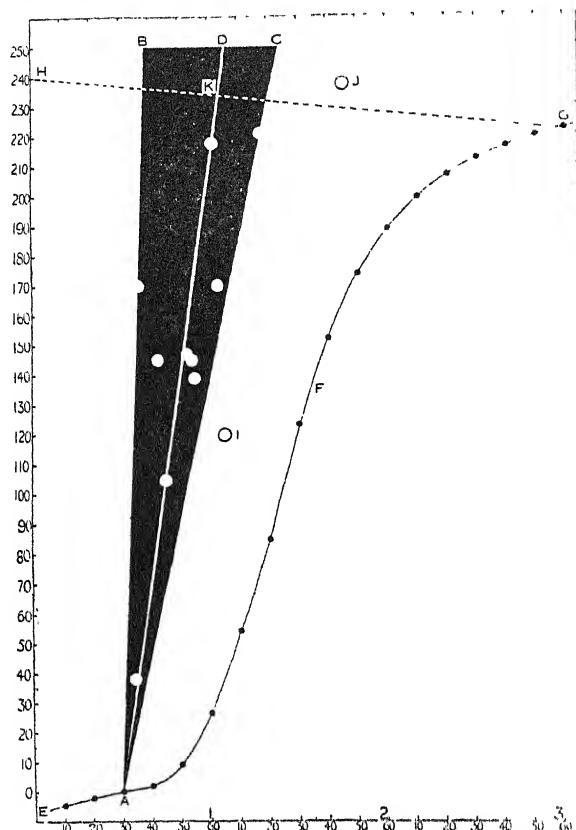


FIG. 8.—E, A, F, G = the mean of nine curves from the nine animals referred to in fig. 7, each curve representing the mean volumes of urine secreted by one animal, and plotted against time. The water was given at A. Each point in the black area represents the volume of water absorbed from the gut of a dog in the allotted time, and the line AKD is the mean of these, constructed on the assumption that the volume absorbed is a linear function of time. I and J are anomalous absorption points (see text). HKG = extra-renal loss of water. Ordinate = cubic centimetres. Abscissa = time in minutes and hours.

assume that absorption of water was in this case slow to a degree below the physiological minimum. The latter point, J, almost certainly represents complete absorption.

The exact shape of the absorption-curve is, of course, not determinable from the small number of observations made, but it is not without interest to see

that it reaches the 250 c.c. line in 35½ minutes' time, a figure which agrees closely with the 36 minutes already recorded as that to which an examination of previous data points.

(d) *The Extra-renal Loss.*—It will be observed that the mean volume-curve, EAFG, fig. 8, does not reach the 250 c.c. level at a time when the rate of urinary flow has returned to its original resting value, see E, fig. 7; the dogs excrete in the course of the 3 hours, on an average, 28 c.c. less than the extra water given. In order to find an explanation for this the following experiment was performed.

A dog, previously operated on as already described, was placed in a Pavlov stand mounted on a balance, in a room in which the temperature and pressure of aqueous vapour were automatically maintained at the desired levels.* The course of the experiment is represented graphically in fig. 9. The dog

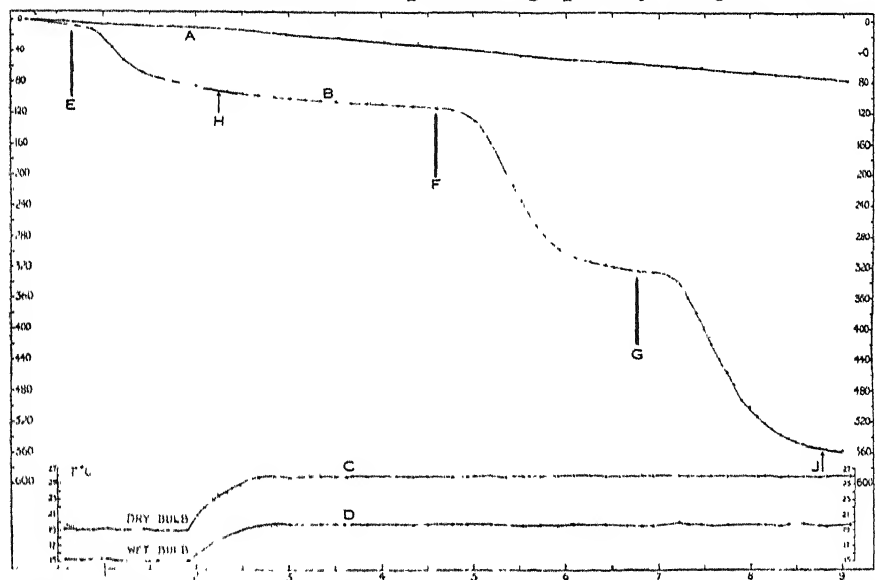


FIG. 9.—Dog No. 65. A = extra-renal loss of weight in grams. B = renal loss of water in cubic centimetres. C = dry bulb temperature and D the wet bulb temperature of the air in °C. At E 100 c.c. water were given by stomach-tube, at F 250 c.c., and at G a further 250 c.c. Abscissa = time in hours. Ordinate = grams and cubic centimetres.

* The room alluded to is one which has been constructed in the laboratories of the Medical Unit at University College Hospital Medical School, for the purpose of investigating problems connected with the rate of water-loss by man, under controlled conditions of atmospheric temperature and humidity. The room, with the results of the investigation for which it was primarily designed, will be described by Dr. D'Arcy Hart and one of us (E. B. V.) in a later publication, but we should like to take this opportunity of expressing our very warm thanks to Professor Elliott for facilities and equipment so generously placed at our disposal.

was given a preliminary dose of 100 c.c. warm water by stomach-tube at E, and its weight to the nearest 0.25 g. determined at intervals of 15 minutes. Its rate of urinary secretion was also measured. The giving of the water does not affect the pulmonary loss, A, fig. 9, which remains steady, for the last 7 hours of the experiment, at 10 g./hour. The giving of the first 250 c.c. was associated, on the part of the kidneys, with an excretory deficit of 20 c.c. more water than was that of the second, a fact which one is tempted to correlate with the prolonged period of abstinence from water preceding the first dose of 250 c.c. The dog is, then, running into water-debt under the conditions of our experiment, and the magnitude of this can be determined if we make one assumption, an assumption which appears to us to be reasonable. We assume that at equal rates of urinary flow, during the ebb of the water-diuresis curves, the dog as a whole is hydrated to the same degree. The rate of urinary flow at H is the same as at J, but between H and J the dog has failed to excrete through its kidneys all the water given it in this time, the average deficit being 6 gm. per hour. It is losing weight through the pulmonary tract at a constant rate of 10 g. per hour, and since, in the dog, the amount of heat lost in vaporization of water is about 70 per cent. of the total (Rubner, 1893), no very great error will be introduced if we take this 10 g. as being wholly water. The magnitude of this error will, of course, depend on the respiratory quotient, but its theoretical extremes are only +0.9 per cent. and -7.5 per cent. for respiratory quotients of 0.71 and 1.0 respectively. The animal, then, is losing water from the pulmonary tract at a constant rate of 10 g. per hour, and is yet, by hypothesis, in the same state of hydration at H as at J. It follows that the difference between 10 and 6, viz., 4 g., represents the water liberated by combustion, and this, in fact, corresponds closely with the quantity which would be expected, at a respiratory quotient of 0.85 (Zuntz and Loewy, p. 663, 1909), to be associated with a vaporization loss of 6000 calories per hour, the actual loss as measured in this experiment. This conformity of reasoning with experience lends support to our assumption, and we have applied the small rate at which we believe this animal to have been running into water debt, viz., 6 gm. per hour, as a correction to our mean excretion-curve. It is shown at HKG in fig. 8. We have used this rate rather than one obtained from the even distribution of the final deficit at G over the period AG, fig. 8. It is unlikely that the mean rate at which a water-debt was incurred in our series of dogs, was greater than the one shown in the figure, as the room-temperature was lower in the former experiments (see the initial slope of the curve A in fig. 9). Our series of observations included responses to the first

dose of the day, so the mean initial debt at A, viz., 13 c.c., represents, with reason, the mean degree to which the animals were below the physiological optimum with respect to the content of their tissues in water. The correction HKG is a small one, and does not affect the nature of the conclusions which we shall draw.

In this particular experiment the rate of incurring the water-debt was not uniform with time. It may be that the giving of water is followed by a qualitative change in metabolism, and if so, we might expect this change to have an alteration in the rate of elimination of water as one of its manifestations. This aspect of the problem, however, is more open to satisfactory solution in the human subject. Carpenter and Fox (1930) state that in man "amounts of water over 200 c.c. work for a definite increase in the metabolism of carbohydrate." Their figures seem to us hardly qualified to substantiate this conclusion.

(e) *The Water-load Curve, and its Relation to the Rate of Excretion of Water by the Kidney.*—Subtraction of the corrected water-excretion curve from the water-absorption curve, or subtraction of AFG from AKG, fig. 8, will give us a curve which represents the amount of water temporarily held in the tissues in excess of the optimal. This curve, the water-load curve, is shown at BCDE in fig. 7, and, as will be clearly seen, its peak precedes that of the curve FGHE, representing the mean rate of urinary secretion, by 15 minutes. If we regard the water-load curve as tracing the intensity of the stimulus to which the kidney responds, we must conclude that there is a lag of 15 minutes between the maximum intensity of stimulus, and the maximum response by the kidney. From the figure it will be evident that the water-load curve is falling when the rate of urine-flow is still advancing, and that, by the time the latter has reached its peak, the former has already fallen by as much as 24 per cent. of its maximum value. We have already referred to Rioch's work on the electrical conductivity of serum following water-ingestion, and it is of interest here to compare with our own findings the conclusion at which he arrived, viz., that there was a lag, about 15 to 20 minutes in duration, of diuresis behind the changes in concentration of the serum-electrolytes.*

The question of the relationship of the curves representing water-load and rate of urine-flow has also been attacked in a slightly different way. The absorption-curve led us to anticipate that 150 c.c. water, given by mouth,

* Since the above was written a paper has appeared by Heller and Smirk ('J. Physiol,' vol. 76, p. 1 (1932)), in which it is shown that "in the guinea-pig and in the rat alimentary absorption is well in advance of diuresis."

would be absorbed in 22 minutes. The response of one of the dogs to the giving of 150 c.c. water was found, a second and equal dose then given, and the dog killed 22 minutes later. Five c.c. only of watery fluid were found in the gut, including traces of clear water in the ileum; so we may conclude that absorption was, at least, just completed. The responses are depicted in fig. 10, from which it will be clear that the peak of diuresis was not to have been expected, had the dog lived, until 13 minutes later. The results of another experiment, done in a precisely similar way, are given in fig. 11, and here 3 c.c. only of fluid were found unabsorbed. In this experiment the latency was 17 minutes, and both this and the 13 minutes found in the previous experiment, accord well with our mean latency of 15 minutes. It is of interest to see that in fig. 10 the final response was succeeding, and in fig. 11 preceding, the initial

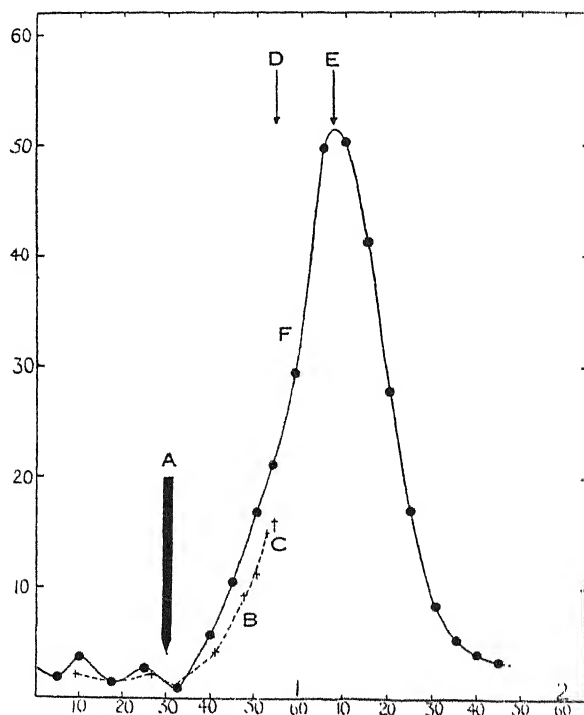


FIG. 10.—Dog No. 53. A = time and duration of the giving of 150 c.c. water by stomach-tube. F = response to initial dose of water. BC = beginning of response to final dose. Dog killed at CD. E = peak of initial response. Abseissa = time in minutes and hours. Ordinate = rate of urinary flow in cubic centimetres per 15 minutes.

response, observations which may well account for the sign of the variation in each experiment from the mean figure previously obtained.

It is important to realize that, since we were interested to know primarily if a latent period between absorption and excretion did, in fact, exist, precautions were taken to ensure that the figures, from which it was deduced were so chosen as to give it a negative rather than a positive bias. As has been stated, the time-element in the absorption-curve is the interval between the middle of the water-giving and the time of puncture of the heart. It

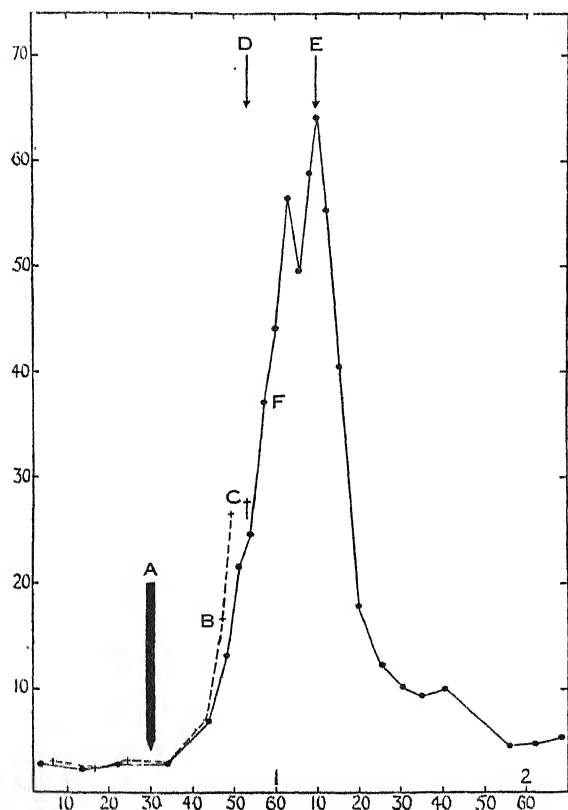


FIG. 11.—Dog No. 52. The letters have the same significance as those in fig. 10.

seems reasonable to us to suppose that the administration of chloroform during the last 2 to 2½ minutes of the interval, delayed, rather than accelerated, the absorptive processes. This being granted, each absorption-point, to represent reality in this regard, should be displaced slightly to the left of the position given it in the figure. Again, in the construction of the curves giving the rates of excretion, the time of each plotted point is the mid-time of the interval during which the sample of urine is collected. The curves will thus tend towards a displacement to the left of their true position while the urine shows

a positive acceleration, and to the right, with a negative acceleration. At the frequencies at which the urine-samples were collected in our experiments, however, this theoretical displacement is too small to be of significance. It may be stated with confidence, therefore, that the mean figure, 15 minutes, obtained under the conditions of our experiments, does not represent too high an estimate of the interval of time clapsing between the peak of the water-load and that of the rate of urine-flow.

(f) *Disparity in the Responses of the Two Kidneys*.—There is observed not infrequently a disparity in the courses of the responses of the two kidneys, that of the right preceding or succeeding that of the left. The phenomenon is to be observed in fig. 1, and is commonly much more in evidence when the responses are small and delayed as the result of the tissues being hydrated to a subnormal degree. In any one animal precedence in time is not always taken by the response of the same kidney; consecutive responses, even, may show a change from right to left, or *vice versa*, in this regard. These facts demand the ascription of at least part of the total delay between the absorption and excretion of water to processes located within the kidney.

(g) *The Response of the Denervated Kidney to Water-Ingestion*.—We propose to limit our remarks to a description of the effects of section of the renal nerves on the response of the kidney to water-ingestion. Frey (1907) cut these nerves on the left side in a rabbit under ether anæsthesia, and, after allowing the state of anæsthesia to subside, introduced water into the stomach. No response, other than a fall in the specific gravity of the urine from the left kidney, was recorded, and Frey accepts this as sufficient proof that water-diuresis is not of central nervous origin. Motzfeldt (1917) gave a more satisfactory demonstration of this, in rabbits, by combining the section of one splanchnic nerve with the removal of the contralateral kidney, and, one to eight days later, determining the response of the animal to the introduction of water into its stomach. A diuretic response was obtained, but the method forbade any conclusion as to whether, or no, section of the renal nerves produced a change in the course and degree of this response. An answer to this question was given in the papers of Weir, Larson and Rowntree (1922) and Bykow and Alexejew-Berkmann (1931). Weir, Larson and Rowntree showed that normal diuretic curves were obtained as the response to water-ingestion in a series of dogs after section of the “splanchnic nerves to both kidneys.” A more convincing demonstration, in that it involved *simultaneous* comparison of the responses of the innervated and denervated kidneys, was given by Bykow and Alexejew-Berkmann. They brought the ureters of dogs to the

surface by a modification of Pavlov's (1883) method. Later, the nerves at one hilum were destroyed as completely as possible, and, some days later, the responses of the two kidneys to water-ingestion determined. Fig. 4 of their paper shows these responses to be closely parallel. We have been able to confirm these results by the method already described. The animal, whose responses are shown in fig. 12, was operated on on May 14, 1930. The operation consisted in extending the ureters to the surface, and dividing the left

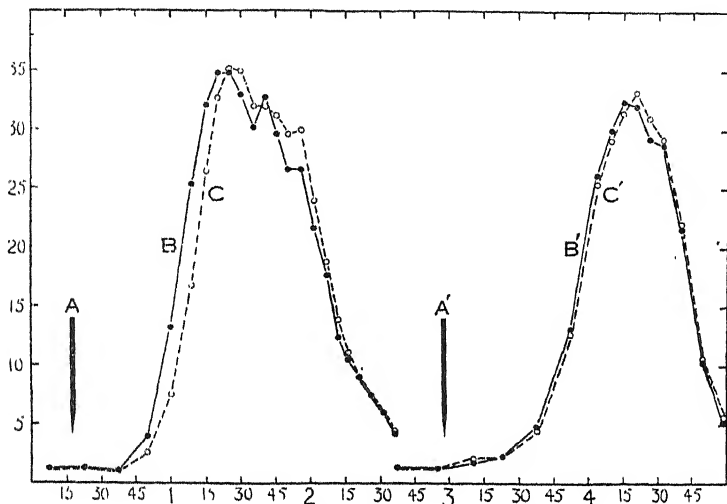


FIG. 12.—Dog No. 37. The responses of the kidneys to the giving of water after section of the left splanchnic nerves. Nerves divided May 14, 1930. B = response of right kidney, C — that of left, on May 16, 1930. At A 280 c.c. water were given by stomach-tube. The responses were again obtained on May 20, 1930, and are shown at B' and C', 300 c.c. being given at A'. Abscissa = time in minutes and hours. Ordinate = rate of urinary flow in cubic centimetres per 15 minutes.

major and minor splanchnic nerves immediately above the suprarenal gland. On May 16, 1930, the dog was given 280 c.c. water by stomach-tube. The responses of the right and left kidneys are shown at B and C, respectively. Four days later, a further dose of water was given, and the corresponding responses are shown at B' and C'. The response preserves its normality even when elicited as early as 15 minutes after division of the splanchnic nerves. This was proven by using the Bowden cable technique (Klisiecki, Pickford, Rothschild and Verney, 1931) as a means of functional division of the left splanchnic nerves in the conscious animal. The possibility that the efferent autonomic nerves to the kidney subscribed any essential part to the response which we are considering, is, we believe, made unlikely, almost to the point

of exclusion (*cf.* Cohnheim and Roy, 1883). by the fact that careful division of all visible nerves on and near the renal vessels and the ureter, leaves the response unaffected, fig. 13.

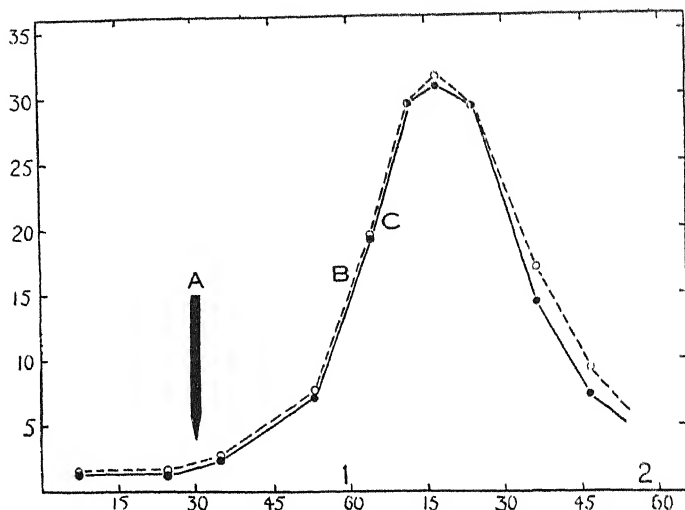


FIG. 13.—Dog No. 43. The responses of the kidneys to the giving of water after dividing the left splanchnic nerves and all visible nerve twigs passing to the left kidney. The nerves were divided on May 4, 1931. The responses shown were obtained on May 14, 1931. B = response of left kidney, C = that of right. At A 250 c.c. water were given by stomach-tube. Abscissa and ordinate as in fig. 12, *q.v.*

We have already referred to the work of Weir, Larson and Rowntree, who obtained normal diuretic responses to water from dogs in which “the splanchnic nerves to both kidneys” had been divided. We decided to repeat these experiments for two reasons: first, the important theoretical bearing which the result has on the processes to which the response of the kidney may be objected; and second, the aid which such experiments offer to the correct interpretation of the effects of exercise on the response. The ureters of a dog (No. 45) were, therefore, extended to the surface, the right splanchnic nerves divided, and a fine Bowden cable was placed in position (Klisiecki, Pickford, Rothschild and Verney, 1931) so that functional division of the left splanchnic nerve could be carried out, at a later date, in the conscious animal. Observations were made the day after this division and normal responses to water were obtained. This fact demonstrates that the splanchnic nerves play no essential part in the phenomenon, even as a path for afferent impulses which might hypothetically be concerned in its production (*cf.* Bradford, 1889, 1898).

In order to exclude completely the possibility of the renal nerves acting as the pathway of afferent impulses essential to the response we are considering, it would be necessary to show that the response remained after either complete denervation of both kidneys, or complete denervation of one and excision of the other. As a result of the work already described, however, the contingency appeared too remote to prompt us to enforce its exclusion. Moreover, we have since become acquainted with an experiment made by C. and M. Oehme (1918) in which a response to water was obtained in a rabbit, both kidneys of which had been previously denervated.

In all the experiments recorded in this paper in which the splanchnic nerves were divided, the division was confirmed at the post-mortem examination (animals Nos. 37, 43 and 45). In animal No. 45, the left nerves were found crushed by the cable so as to leave, from anatomical inspection alone, very little doubt as to the complete loss of their conductive function. This loss was demonstrated by opening the chest under chloralose-anæsthesia and artificial respiration, ligating and sectioning the splanchnic nerve on each side about 5 cm. above the crura of the diaphragm, and stimulating the peripheral ends with induction shocks. Blood-pressure was recorded from the left carotid artery. In all cases so examined, in which the nerves were intact below the diaphragm, the blood-pressure trace showed the usual characteristics, whilst in those in which the nerves had been divided or crushed below the diaphragm, the trace remained perfectly steady during the period of stimulation.

In several of our experiments we have analysed the secretions of the innervated and denervated kidneys before giving the water, and at the height and subsidence of the diuresis. Our data are abstracted in Tables I and II. It will be seen that the giving of water does not, as a rule, produce any marked change in the output of chloride, a fact which agrees with observations on man to the effect that the excretion of water is largely independent of that of chloride (Haldane and Priestley, 1916; Motzfeldt, 1917; Priestley, 1916, 1921; Brunn, 1920; Adolph, 1921; Baird and Haldane, 1922; Crawford, 1927).

On four occasions we noticed a definite rise in the percentage of chloride in the urine from both the innervated and denervated kidneys, immediately following the administration of water. As this might be observed before the onset of the diuresis, it seemed probable that it was owing to transitory interference with the venous return from the kidney (Theobald, 1933) by the increased bulk of the stomach. A temporary inhibition in the output

of water is usual directly after the water has been given (see the curve EAFG, fig. 8).

Further, it will be observed that denervation of the kidney does not affect significantly the chloride-content of the urine secreted in response to the giving of water.

Published work and the experiments described above have led us to the view, therefore, that the phenomenon of water-diuresis, with its associated change in the concentration of urinary chloride, is conditioned independently of the nervous supply to the kidneys.

Table I.

	Right kidney.	Left kidney.
Urine in cubic centimetres/15 minutes	3.3	3.5
Cl' milligram/100 c.c.	112.0	115.0
Cl' milligram/15 minutes	3.7	4.0

The figures show the effect of section of the left splanchnic nerves, or denervation of the left kidney, on the rate of flow and the chloride-content of the urine. They are the mean of nine observations on six animals, the observations being made at times varying from 20 minutes to 23 days after functional or structural division of the nerves. Chloride is expressed as NaCl.

Table II.

	Before diuresis.		At height of diuresis.		At fall of diuresis.	
	Right kidney.	Left kidney.	Right kidney.	Left kidney.	Right kidney.	Left kidney.
Urine in cubic centimetres/ 15 min.	2.9	3.3	29.5	26.3	7.5	8.2
Cl' milligram/100 c.c.	188	225	19	19	79	85
Cl' milligram/15 minutes	5.5	7.4	5.6	5.0	5.9	7.0

The figures show the effect of the giving of water on the chloride-outputs of the innervated (right) and denervated (left) kidneys. They are the mean of three observations on two animals, the observations being made from 1 hour to 9 days after functional or structural division of the nerves. Chloride is expressed as NaCl.

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IV. *Summary.*

(1) An analysis of data in published work relevant to water-absorption, suggests that 250 c.c. are absorbed by the small gut of a dog weighing 10 kg. in a period of 36 minutes.

(2) The time-course of water-absorption by the normal dog has been traced experimentally, and a mean figure obtained, which agrees closely with the conjectural period, 36 minutes, given above. This figure is demonstrated to be 35 minutes.

(3) Through the use of a method which allows the secretion of each kidney to be collected separately, the time-course of the responses of the kidneys to the introduction of 250 c.c. water into the stomach, has been followed. From two curves, one of which represents the time-course of the quantity of water absorbed, the other that of the quantity of water excreted by the kidneys and respiratory tract, a third curve has been constructed to show the time-course of the extra-water held in the tissues. The peak of this curve, the water-load curve, is found to precede the peak of the curve representing the rates of water-diuresis, by an interval of 15 minutes. The water-load curve has already fallen to 76 per cent. of its maximum, by the time the rate of water-excretion has reached its highest value. Confirmatory evidence also for a minimum latency of 15 minutes has been obtained by another method.

(4) There is observed, not infrequently, a disparity in the course of the responses of the two kidneys, that of the right preceding or succeeding that of the left. In any one animal precedence in time is not always taken by the response of the same kidney; consecutive responses, even, may show a change from right to left, or *vice versa*, in this regard. Part of the delay between the absorption and excretion of water is, therefore, located within the kidney.

(5) Division, as complete as has been found possible, of the nerves on the pedicle of one kidney, does not affect the parallelism between the responses of the two kidneys to water-ingestion (confirming Bykow and Alexejew-Berkmann, 1931).

The possibility of the renal nerves contributing essentially to the response, in the capacity of an afferent pathway, is made unlikely by the fact that bilateral section of the splanchnic nerves does not affect the course of the water-diuresis curves. The giving of water does not appreciably alter the rate of chloride-excretion (confirming Priestley, 1921). This is still true after denervation of one kidney, and the rates of chloride-excretion then retain a close parallelism on the two sides.

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*The Absorption and Excretion of Water by the Mammal. Part II.—
 Factors Influencing the Response of the Kidney to Water-
 Ingestion.*

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I. *Introduction.*

In Part I the results were reported of experiments on the relation between absorption of water by the gut and its excretion by the kidney. Demonstration was also given to the independence of excretory response and innervation of the kidney. In the present paper, use being largely made of the method detailed in Part I, our object will be to describe the effects of some factors influencing this response, and finally to select the hypothesis which, on the one hand, may best embrace the facts of this and previous work of a relevant nature, and, on the other, serve as a fruitful source and guide for future investigation.

II. *Results of Experiments.*

(a) *The Effect of Exercise on the Responses of the Innervated and Denervated Kidneys to Water-ingestion.*—The inhibition, by exercise, of the response of the kidneys to water-ingestion has been commonly ascribed to a concomitant

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diminution in the supply of blood to the kidney, either as the passive result of redistribution of capacity in the vascular system, or as the result of an increased activity in the vaso-constrictor nerves. The possibility of its being due, through similar causes, to an inhibition of absorption by the gut must also be considered. MacKeith, Pembrey, Spurrell, Warner, and Westlake (1923), in a paper dealing with the adjustment of the human body to muscular work, report some observations on the effect of exercise on the diuretic response to 560 c.c. hot tea taken by mouth. They were led, by the results of their experiments, to suggest that the suspension of the activity of the kidneys during running arose from an early outflow of constrictor impulses to the renal vessels.

The operative procedure already described afforded an adequate basis for testing the hypothesis which would ascribe to the renal nerves an essential rôle in the inhibition, by exercise, of the response of the kidneys to water-ingestion. Dogs in which the left splanchnic nerves had been divided previously, with or without section of all visible twigs on the renal pedicle, and the ureters extended to the surface, were placed in Pavlov stands, and the responses of each kidney to the giving of 250 c.c. water by stomach-tube determined. At varying times after the water had been given, the dogs were freed from the stands, run around the laboratory or the roof of the building, and then rapidly replaced. The exercise was mild, and in no case did panting supervene. During periods of 3 or 4 minutes before, and after, exercising the animal, the rates of flow were determined by measuring the times of collection of certain numbers of drops of urine. Failure to observe transient changes in rate was thus combated. We have collected our data in Table I. From these it is clear that, following exercise, the rate of urine-flow from the denervated kidney, or from the kidney on the same side as that on which the splanchnic nerves have been divided, is inhibited to a degree at least equal to that to which the flow from the kidney with intact nerve-supply is inhibited. These facts are illustrated graphically in figs. 1 and 3. We feel that it would be unreasonable to attribute to the few intramural fibres which may have escaped division, any causative relation with the phenomenon of inhibition which is under consideration. The executive cause must lie elsewhere than in the renal nerves.

The possibility of the inhibition being due to a local fall in pressure in the renal arteries, resulting from an increase in the velocity of the blood in the aorta, is made improbable by the synthesis of the following circumstances. First, the inhibition is accompanied by a rise in the concentration of chloride in

the urine, figs. 1 and 2, an association to which constant experience fails to object a fall in blood pressure, *per se*, as an adequate cause. Second, and interrelated with this, the type of diuresis is that to which our experience with the isolated perfused kidney ascribes a high grade sensitivity to humoral environment, and a low grade sensitivity to such variations in the mechanical conditions of perfusion as can be reasonably expected to occur in the living

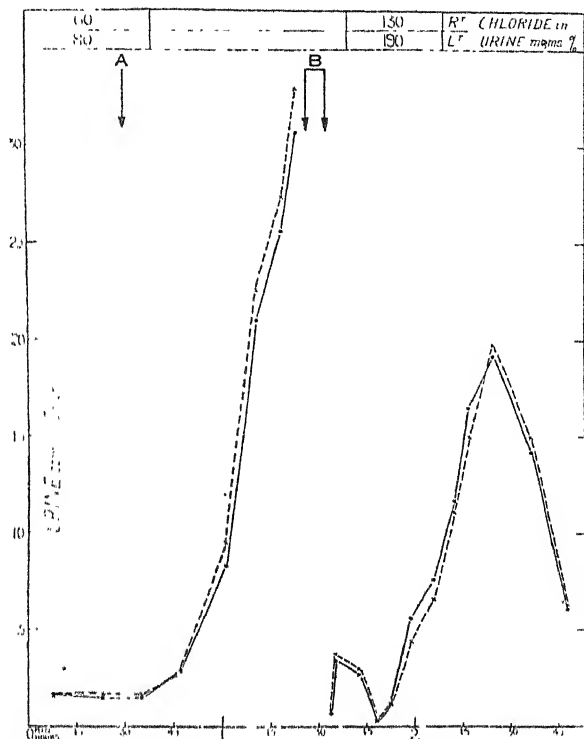


FIG. 1. Dog No. 38. The effect of exercise on the responses of the kidneys to the giving of water. The left splanchnic nerves and all visible twigs at the origin of the left renal artery had been divided 4 days previously. At A 300 c.c. water were given by stomach-tube. During the interval B the animal was run around the laboratory. The amount of chloride (as milligrams NaCl per 100 c.c. urine) is given at the top of the figure. Abscissa = time in minutes and hours. Ordinate = rate of urinary flow in cubic centimetres per 15 minutes. — Rt., - - Lt. kidney.

animal, in the absence of local vasomotor control. Changes of 20 mm. Hg, for example, in the physiological range of arterial pressure, may produce 100 per cent. change or even less in the rate of urine-flow from the dog's kidney perfused with a heart-lung preparation. Third, it seems reasonable to ascribe the inhibition we have observed in the dog to the same co-ordinated changes as underlie this phenomenon in man. And here, as was shown by MacKeith,

Pembrey, Spurrell, Warner, and Westlake, "a feigned run for 3 or 4 minutes, during which the tense attitude for starting to run was maintained, produced a definite decrease in the urine . . ." This fact, and the mildness of the exercise undertaken by our dogs, also make it difficult to believe that anoxæmia bears even a contiguous relation with the phenomenon under discussion.

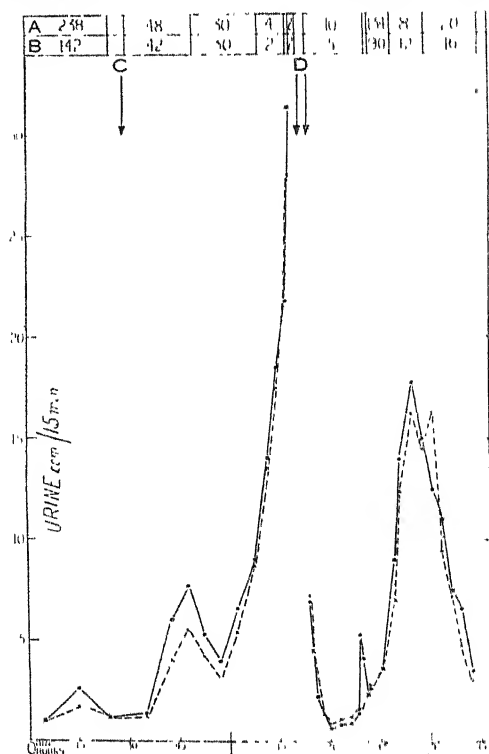


FIG. 2.—Dog No. 45. The effect of exercise on the responses of the kidneys to the giving of water. May 29, 1931, right splanchnic nerves divided, and Bowden cable put down to left splanchnic nerves. June 1, 1931, left splanchnic nerves crushed under local anaesthesia. June 2, 1931, 250 c.c. water were given at C by stomach-tube. During the interval D the animal was run around the roof of the building. A and B: amounts of chloride (as milligrams NaCl per 100 c.c. urine) in the urine from the right and left kidney respectively. Abscissa and ordinate as in fig. 1. — RT. kidney. --- Lt. kidney.

It remains to consider the possibility of the inhibition being caused by a diminished rate of absorption of water by the wall of the gut, a change theoretically attributable to nerve-influence exerted on the processes of absorption, either directly or indirectly, through the local vascular bed. In order to bring such an hypothesis to the test of sense the following experiment was performed. At the operation during which the ureters were extended to

the surface, the right major and minor splanchnic nerves were divided immediately behind the upper part of the right suprarenal gland. A fine Bowden cable was then sutured behind the left kidney, and so arranged that the left splanchnic nerves could be functionally divided later, under local anaesthesia in the conscious animal (Klisiecki, Pickford, Rothschild, and Verney, 1931). The operation was necessarily a long one, but recovery was rapid. Fifteen hours after the operation, the animal appeared to be in excellent condition, and observation of the urine showed the rates to be approximately equal on the two sides. Periodic observation showed that this equality persisted over the next 48 hours, at the end of which time functional discontinuity in the left splanchnic nerves was effected under novocaine anaesthesia. The following day, the observations graphically recorded in fig. 2 were made. These observations show that the conjecture that inhibition of urine-flow is caused by retardation of absorption, effected through the intermediation of the splanchnic nerves, is not in agreement with facts.

The assignment of the inhibition of secretion to interference, by any process, with so early an event as absorption is controverted, as we believe to complete disproof, by the following considerations. We have already shown that the absorption of 250 c.c. of water is complete 35 minutes after its introduction into the gut, fig. 8, Part I. This was a mean figure of 10 observations, and that it was not an underestimate, was indicated by the two experiments to which figs. 10 and 11 of that paper relate. The maximum period of absorption found in our series, was 50 minutes. Now exercise has usually been imposed at times later than the end of this period (see Table I), and has been invariably followed by a reduction in the rate of urine-flow. In figs. 1 and 2, for example, we see that diuresis is following its usual course up to 55 and 52 minutes, respectively, from the time of giving the water, and that exercise then brings about a profound inhibition of secretion. We conclude that exercise will inhibit the diuresis following the giving of water at times when absorption is complete. The inhibition is, therefore, due to an involvement of one or more of the later events which underlie the secretory response.

There are two further points to which we should like to refer whilst dealing with the influence of exercise on water-diuresis. First, the minimum rate of urine-flow is not reached till several minutes have elapsed. We have been unable to follow the rate during the actual period of exercise, and so cannot say whether, or no, the observed progression in the fall represents a continuation of a process already in existence. As will be seen from the table, the minimum rates which were recorded in the 11 observations given, occurred at times varying

Table

No. of animal.	Time at which water was given. Minutes before exercise.	Time in minutes before exercise.									Period of exercise (min.).	Time in						
		10	9	8	7	5	4	3	2	1		1	2	3	4	5	6	7
32	127					15.1 14.4				12.0 12.1	20		3.0 3.0		3.0 3.0			2.1 2.7
35	45	18.6 24.3			22.3 27.6		27.0 30.0		30.0 30.0	25.5 25.5	18	7.5 6.3	5.4 5.4	4.9 4.5	4.9 4.9			
36	55									16.5 22.5	4	16.5 22.5				10.2 8.1		
	88				13.8 13.2					12.7 12.0	10	10.5 8.1	9.0 5.4		7.2 5.4	5.4 5.6		1.1 2.9
37	80	22.8 22.2					21.0 16.5		21.0 15.0	21.0 15.0	10	13.5 15.0		13.5 12.0		12.7 12.0		
38	55			25.6 27.4			30.7 33.0			27.0 31.5	6	0.7 0.7	3.4 3.7					
43	66	23.4 21.3				34.2 29.7			39.0 37.5	49.0 38.0	4	27.0 24.0		13.8 12.9				
	33								7.5 9.9	8.0 10.2	5	9.6 7.5	7.0 7.0		2.6 3.6			
	75			8.1 10.6					15.0 15.0	15.0 15.0	5	4.0 0.6	1.4 0.0				1.4 2.2	
44	49		17.9 16.4					25.0 29.0	22.0 31.3	31.3 32.6	4	14.4 13.3			11.6 9.5		11.2 13.3	
45	52			14.0 13.5		18.5 17.5		21.8 23.2	25.0 25.0	26.0 26.0	2½	6.9 7.2	4.5	2.1		1.3		0.6 0.9

The table shows the effect of exercise on the rates of flow of urine from the two kidneys during diuresis following pair of observations is the rate of urine-flow from the right, the lower that from the left, kidney. The minimum

minutes after exercise.															Remarks.	
9	10	11	12	13	14	16	17	18	19	20	23	24	26	28	30	
					1.0 4.2						4.0 4.0					Renal nerves intact.
			5.1 5.1									6.0 6.0				Renal nerves intact.
		14.2 15.7														Left splanchnic nerves divided.
			5.4 6.6													" " "
			15.6 15.6													Left splanchnic nerves divided.
2.7 3.0					0.3 0.3				1.2 1.2				5.6 4.4			Left splanchnic nerve and nerve twigs around left renal artery divided.
	12.0 19.2													13.8 15.4		Left splanchnic nerve cut. Left kidney denervated.
			1.9 3.6							3.6 9.6					8.1 10.6	" " "
								1.5 2.7							4.1 5.7	" " "
	13.3 15.0							21.4 23.2								Left splanchnic nerves crushed.
	0.8 1.1			0.9 1.2	1.5 1.3	6.6 1.6	2.3 2.4	2.7 2.9			3.6 3.6		9.0 7.0	14.0 12.4		Right splanchnic nerves divided. Left splanchnic nerves crushed.

the giving of water by stomach-tube. The figures are in cubic centimetres per 15 minutes. The upper of each rates observed are italicized. The horizontal lines separate observations on one animal from those on another.

from 2 to 14 minutes after the ends of the periods of exercise, the density of these minima being greatest from the third to the sixth minute. Denervation of the kidney is without apparent influence on this result. The second point is that the fall in the rate of flow is accompanied by a rise in the concentration of chloride in the urine, figs. 1 and 2. The possible significance of these two points will be apparent later, p. 539.

The collation of the facts recorded in this section leads to the conclusion that the inhibition of water-diuresis by exercise is the expression of a direct action on the kidney of some change in the composition of the blood, a change which is independent of any possible diminution in the rate of absorption by the gut. As to the essential nature of this change, a suggestion will be put forward when we come to discuss the results of this work in their relationships with one another.

In all the experiments recorded in this paper in which the splanchnic nerves were divided, the division was confirmed at the post-mortem examination (animals Nos. 36, 37, 38, 43, and 45). In animals Nos. 44 and 45 the left nerves were found crushed by the cable so as to leave, from anatomical inspection alone, very little doubt as to the complete loss of their conductive function. In animals Nos. 38, 44, and 45 this complete functional breach was demonstrated by opening the chest under chloralose-anæsthesia and artificial respiration, ligating and sectioning the splanchnic nerve on each side about 5 cm. above the crura of the diaphragm, and stimulating the peripheral ends with induction shocks. Blood pressure was recorded from the left carotid artery. In all animals in which the nerves were intact below the diaphragm the blood pressure trace showed the usual characteristics, whilst in all those in which the nerves had been divided or crushed below the diaphragm, the trace remained perfectly steady during the period of stimulation. In No. 38 the effects on the urine-flow of stimulating the nerves were also observed. The rise in blood pressure produced by stimulation of the peripheral end of the right splanchnic above the diaphragm was accompanied by a fall to zero in the rate of urine-flow on the right, and by a quickening in the rate of the left, side. Stimulation of the peripheral end of the left splanchnic above the diaphragm was without influence on the rate of urine-flow.

In one observation made on dog No. 36, the left splanchnic nerves having been cut 2 days before, an attempt was made to see whether an emotional disturbance would affect the responses of the kidneys to water-ingestion. To this end a cat was introduced, and held before the dog during the rising diuresis. A temporary fall in the rate of urine-flow ensued, the fall being about equally marked on the two sides. We have made no attempt to analyse this phenomenon.

(b) *The Effect of Post-pituitary Extract on the Responses of the Innervated and Denervated Kidneys to Water-ingestion.*---That post-pituitary extract either delays or checks water-diuresis has been known since the publication of the work of Korschegg and Schuster on rabbits in 1915. This result has since been confirmed by Motzfeldt (1917) (rabbits), Rees (1918) (rabbits and cats), Priestley (1921) (man), Weir, Larson, and Rowntree (1922) (dogs). The delay has been assigned to a variety of sites, the gut, central nervous system and kidney having been severally invoked as the primary seat of the extract's action.

Motzfeldt (1917) accepted the antidiuretic effect, which he observed the injection of pituitary extract to have on the polyuria produced by the giving of saline subcutaneously, as evidence that the action of the extract in water-diuresis was not due to a delay in absorption from the gut. Rees (1918) concluded that the delay in the onset of diuresis "was due in part at least to a delayed absorption from the alimentary canal," but (Rees, 1920) that this did "not seem to be sufficient, in most cases, to account entirely for the delay in the excretion of water from the kidneys." Craig (1925) showed that in the cat and dog it was impossible that a delay in diuresis for 3 to 3½ hours could be due to delayed absorption from the alimentary tract. Whether or no a diminution in the rate of absorption is a contributory cause in effecting the delay, and published work still leaves the question open, there can be no doubt that the inhibition, by the extract, of an established diuresis is completely independent of any simultaneous involvement of the absorptive act. Of this we have convinced ourselves from the results of subcutaneous injection of the extract,* at times when, as we knew from previous experiment, absorption is already complete. Dogs operated on, as has been previously described, and in which the response to 250 c.c. water given by mouth has recurred with mimetic precision, have been injected subcutaneously with doses ranging from 0.33 to 2 units, and at times varying between 52 and 80 minutes after the giving of the water (eight observations). There has resulted the usual inhibition of urine-flow. This is evident about 5 minutes after the injection, and progresses at first rapidly, then more slowly over the next 15 to 20 minutes. We have already shown that the absorption of 250 c.c. water is complete within a period less than the smaller of the two extremes (52 and 80 minutes) given above; the inhibition, therefore, is unrelated to it.

* The extract used in all our experiments has been that marketed by Messrs. Burroughs Wellcome & Co. under the name "Infundin," and standardized in terms of the international oxytocic unit.

In some of his experiments, Motzfeldt (1917) cut the splanchnic nerve on one side and removed the contralateral kidney (rabbits). Observations 1 to 8 days later showed that in some animals pituitary extract had the usual, in others no effect on water diuresis. From this contrariety of results Motzfeldt drew the curious inference "that the splanchnic nerve is the pathway for the normal antidiuretic action of the pituitary extracts." Later, Weir, Larson, and Rowntree (1922) took the response to water of a series of dogs before and after section of the "splanchnic nerves to both kidneys." The normal diuretic curves after section of the nerves were largely abolished by pituitary extract, and the conclusion was drawn "that the influence of pituitary extract in the prevention of diuresis is independent of the nerve supply of the kidney." This result we have been able to confirm on our own preparations. The parallel responses of the two kidneys to the giving of water after unilateral section of the splanchnic nerves, are themselves inhibited to exactly the same degree by a subcutaneous injection of the extract, and this strict parallelism persists after one kidney has been denervated as completely as is found possible, fig. 3. In the experiment to which this figure relates, there was an interval of 11 days between the section of the nerves and the elicitation of the response. Langley and Magnus (1905), in five experiments on the movements of the

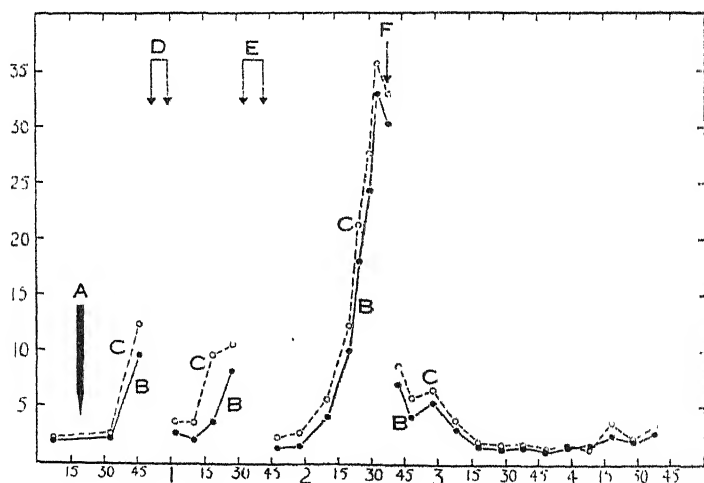


FIG. 3.—Dog. No. 43. The effect of exercise and of post-pituitary extract on the responses of the kidneys to the giving of water. The left splanchnic nerves and all visible twigs passing to the left kidney had been divided 11 days previously. At A 250 c.c. water were given by stomach-tube. B and C = responses of right and left kidneys respectively. During the periods D and E the animal was run round the roof of the building. At F 0.33 unit of post-pituitary extract was injected subcutaneously. Abscissa and ordinate as in fig. 1.

intestine of cats and rabbits after degenerative section of the mesenteric nerves, allowed 5, 7, 7, 10, and 14 days to elapse after division of the nerves. These periods were taken, on histological evidence, as sufficient to ensure the degeneration of the post-ganglionic fibres. In view of this work, it seems to us probable that, in the experiment recorded above, all the divided post-ganglionic fibres had already undergone complete degeneration. It is impossible to believe, therefore, that the splanchnic nerves can subserve any functional relationship with this action of the extract; and with the additional knowledge (Molitor and Pick, 1924) that the inhibition may still be observed in the presence of ergotoxin, one is led inevitably to the conclusion that the renal parenchyma is itself responding to some change in the composition of the blood following directly, as Oehme and Oehme (1918), Priestley (1921), and Underhill and Pack (1923) have been led to suggest, or indirectly through central nervous system and tissues, as Molitor and Pick (1924, 1925, *a* and *b*, 1926), Mehes and Molitor (1926), and Hoff and Werner (1927, *a* and *b*) have been led to believe. We think the former interpretation to be the correct one for two reasons. First, the many affinities which the response of the kidney to isolation and perfusion with a heart-lung preparation (Starling and Verney, 1925; Verney, 1929) bears to its response to water-ingestion, foster the opinion that the responses are congenetic and not merely resembling; and pituitary extract inhibits the polyuria of the isolated kidney. Second, the results of Molitor and Pick and their associates are neither consistent with one another, when regarded in the light of their own theory, nor with those obtained by some other workers (Janssen, 1928; Fee, 1928 and 1929, *a*). Indeed, a study of the literature has convinced us that no unequivocal evidence exists in support of the hypothesis of Molitor and Pick; rather is it opposed by a large body of experimentally acquired fact. We are of the opinion, therefore, that the inhibition of water-diuresis is the expression of a direct action of pituitary extract on the renal parenchyma.

(c) *The Effect of Thyroxine on the Response of the Kidney to Water-ingestion.*—In a series of 33 hypophysectomies performed by one of us on dogs, and briefly referred to elsewhere (Verney, 1929) it was found that polyuria was not a constant sequela to the removal. In 25 per cent. of the cases, no increase in urine-flow supervened, and no single factor was discovered, to which the absence of response could be attributed (see later under Discussion). There was observed, however, a greater tendency for the polyuria to occur in young, and lively, than in old, phlegmatic animals. This observation, combined with the knowledge that diabetes insipidus is pre-eminently a disease of youth, led to the idea that the degree of activity of the thyroid gland might affect the

intensity of the response of the kidneys to removal of the pituitary body. The knowledge of the many properties which the response of the kidneys to water-ingestion has in common with that to removal of the pituitary body, and an acquaintance with Eppinger's (1917) work on the effect of thyroid feeding on the former, suggested to us the desirability of seeing whether the giving of thyroxine would alter the course of water-diuresis. It should be clearly understood that the experiments to be described in this section, though born of conjecture of physiological interest, are not, in themselves, to be considered as affording evidence for the direct dependence of the kidney on the activity of the thyroid gland. Rather is their interest to be regarded as pharmacological only, and their importance as deriving from the possibility of their affording yet other evidence for the essential identity of water-diuresis with the polyuria following hypophysectomy, and that following isolation of the kidney.

The animals were prepared for the following experiments by perineotomy, since the technique described in Part I did not allow a period of survival sufficiently long. We found the results of the following procedure satisfactory in every way.

Under ether anaesthesia, following induction with chloroform and ether, the vulva is shaved and the skin cleaned with alcohol and ether and finally swabbed with tincture of iodine. A sagittal incision is made through the posterior wall of the vagina below the level of the urethral orifice. The incision passes through the perineum to a point about 2 cm. from the anus. The posterior vaginal wall at the anterior extremity of the incision, is then sutured with silk to the skin at the posterior extremity of the incision. The urethral orifice is now exposed, and an incision, starting 1 cm. below it, is carried forward through mucosa and skin to end over the symphysis pubis. The posterior end of the incision is then sutured to the anterior. By this means the urethral orifice is brought well forward and made clearly visible. Lateral incisions are then made through the vaginal mucosa between the anterior and posterior sutures, and the labia and mucosa below these incisions removed. All hæmorrhage is carefully arrested, the edges of the skin and mucosa approximated with silk sutures, and sterile vaseline applied. The wound heals by secondary intention, and is irrigated twice daily with warm boracic lotion. Healing is complete within about a fortnight, and thereafter the animals can be catheterized with ease to the operator, and with the minimum disturbance to themselves. Rubber self-retaining catheters have been used, and these connected to measuring cylinders placed on the table to which the Pavlov stands are clamped.

Our procedure has been, as before, to give the animals 250 c.c. water by stomach-tube and, half an hour after the urine-flow has returned to a steady

state, to give the test dose of 250 c.c. water. The response to the former dose is discarded, that to the second followed with care. After several such responses have been collected, thyroxine* is injected subcutaneously, the drug being dissolved in distilled water with the aid of a minimum amount of sodium hydroxide. Large doses have been used, the average daily dose per dog being 4 mg. of the sodium salt. Observations on the response to water are continued during the giving of the thyroxine, and following its withdrawal. Sometimes the effects of a further course of thyroxine have been followed. We have observations, made according to the above scheme, on four dogs, and the results obtained from one of them are shown in fig. 4, and may be taken as representative of those from the whole group. In fig. 4 the curve E represents

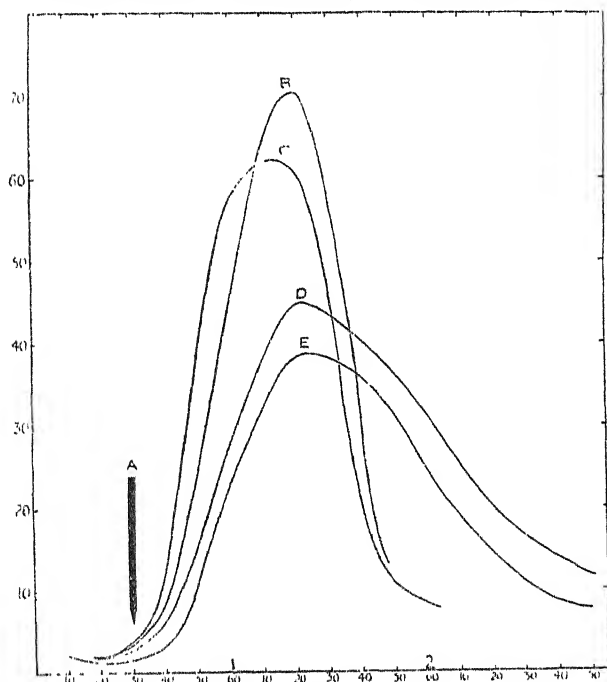


FIG. 4. — Dog. No. B.6. The effect of thyroxine on the course of water diuresis. At A 250 c.c. water were given by stomach-tube. E — mean of six responses before the giving of thyroxine. B — mean of four responses while the animal was under the influence of thyroxine. D — mean of three responses obtained at a minimum of 8 days after the last injection of thyroxine. C — mean of five responses while the animal was again under the influence of thyroxine. The groups of responses of which the means are shown in the figure were obtained in the order and under the conditions given above. Abscissa and ordinate as in fig. 1.

* The thyroxine used in these experiments was given us by Dr. Guggenheim and the Hoffmann-La Roche Chemical Works, Ltd. We should like to express to them our very warm thanks for a most generous supply of the drug.

the mean of six observations over a period of 8 days, before any thyroxine had been given. During the next 10 days 28 mg. of the drug were given to the dog, and four responses obtained between the sixth and tenth day. Their mean is shown at B. A week later, three more observations were made, and their mean, D in the figure, determined. Thyroxine was then re-administered, 33 mg. being given in 6 days, and during this time five more responses were obtained. Their mean is shown at C. The curves show quite definitely a rise in the maximum rate of urine-flow, the duration of the response being diminished and its intensity increased as the result of giving thyroxine.

In fig. 5 are given mean excretion-curves from the four dogs investigated, E being the normal curve, and D the curve obtained from observations when

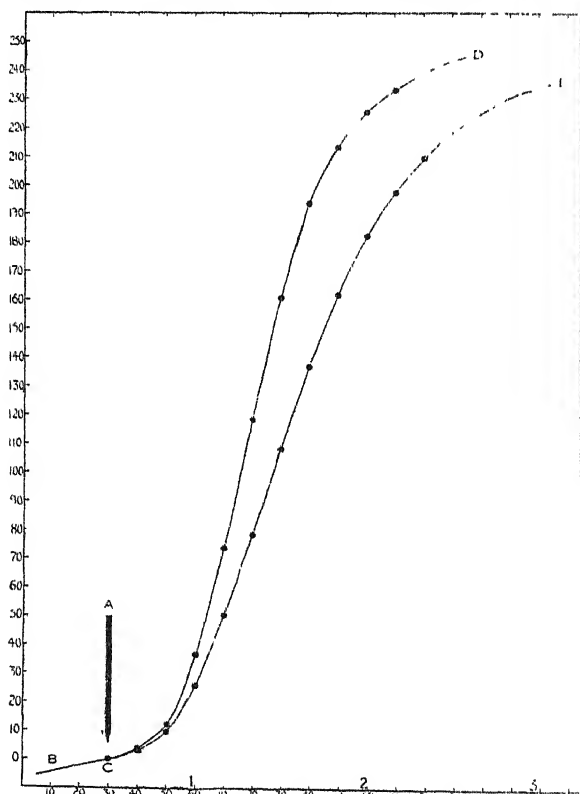


FIG. 5.—Dogs B6, B7, B8 and B10. The mean excretion curves of four dogs taken while they were under the influence of thyroxine, D; and free from this influence, E. To obtain the curve D two to eight curves were taken from each dog, twenty curves in all, and the mean of each group found. The final curve is the mean of these means. The curve E was obtained in a similar way, but two to nine curves were taken from each dog, and eighteen in all. Abscissa = time in minutes and hours. Ordinate = cubic centimetres.

the animals were under the influence of the drug. The components from which these curves were obtained are described in the legend. It will be seen that, on an average, the time required for the elimination of 235 c.c. of urine, is reduced by the drug from 2 hours 30 minutes to 1 hour 40 minutes.

The increased intensity of response was observable 48 hours after the first dose of thyroxine, and the animals often, but not invariably, exhibited increased excitability while they were under the influence of the drug. Our figures give no definite indication that the period between the administration of the water and the maximum rate of urine-flow, is changed, as the result of giving thyroxine.

Eppinger (1917) measured the volume of water excreted by a dog over a period of 3 hours from the time of giving the water, and, after subtracting a volume corresponding to the basal rate, expressed the remainder as a percentage of the volume given. The giving of dry thyroid raised this percentage from about 60 to 100 and over, but the maximum rate of secretion was not appreciably altered. Eppinger attributed his results to an action of the thyroid on the tissues. The simplest interpretation of our own results would appear to be in terms of a direct effect of thyroxine on the kidney, since the outstanding characteristic of its action is an increase in the maximum rate of secretion. A plateau-response becomes peaked in nature, a phenomenon which is not seen, of course, as the result of the mere giving of more water.

III. *Discussion.*

Water-diuresis is a phenomenon to the elucidation of which several hypotheses have been adduced. We propose to confine our attention at first to the one which ascribes to the pituitary body an essential rôle, and to leave consideration and assessment of the others till later.

That the secretion of water by the kidney is normally held in check by a pituitary hormone, the hormone holding the water at, so to say, a higher potential, and by its falling concentration, maintained deficit and reaccumulation in the blood stream, giving rise, continuation and stop to an increased rate of water-loss, is an hypothesis which arose largely as the result of Frank's observation in 1912 of the association of diabetes insipidus with injuries to the hypophysis, an observation which led, in the following year, to the demonstration by v. den Velden and by Farini of the efficacy of pituitary extract in relieving the symptoms of this disease. In 1915 Korschegg and Schuster reported an inhibitory effect of the extract on water-diuresis, a result which was also obtained by Motzfeldt in 1917, and led him (1916, 1917) to the view that the pituitary body exerted "a constant, physiological influence on the

functional activity of the kidneys." This opinion images our own and, we suggest, receives support from work on the isolated kidney of the dog (Starling and Verney, 1925; Verney, 1926) and from the effects of pituitary removal (Vassalle and Sacchi, 1892; Gemelli, 1908; Lewis and Matthews, 1913; Römer, 1914; Verney, 1929).

The hypothesis, imperfectly detailed though it be, has been largely responsible for the experiments described in this paper, and it will here be our aim to proceed by induction and deduction towards confirmation, extension, modification, or abandonment of its central idea.

It seems reasonable to assume that the concentration of water in the blood and tissues, as signified by their aqueous vapour pressure, be the concentration greater or less than the immediate physiological requirement, is the factor responsible for the control of the antidiuretic function of the pituitary gland. The gland may, of course, itself respond appropriately to any change in the concentration of water in its immediate environment, but it seems to us probable that its response is brought about indirectly through the nervous system.

We base this opinion, first, on the work of Marx (1926), Grossmann (1929), Govaerts and Cambier (1930) and Bykow and Alexejew-Berkmann (1930, 1931), in which was shown the possibility of the occurrence of water-diuresis as the result of suggestion in a patient in a state of hypnosis, and as the response to such conditioned stimuli as the passage of a rectal catheter, or the mere placing of the animal in the position and environment in which the unconditioned stimulus has been repeatedly given. A second point which we think suggests the involvement of the nervous system, is the extreme sensitivity of this response to the majority of anæsthetic and narcotic substances, a fact which has been demonstrated by Frey (1907), Ginsberg (1912), Frey and Kumpiess (1913), Motzfeldt (1917) and Fee (1928). Thirdly, there are to be found in the medulla, tracts or centres, the stimulation of which gives rise to a transitory polyuria of the type we are discussing (Bernard, 1855, p. 339; 1858, p. 399; Eckhard, 1869; Kahler, 1886). The *piqûre*-polyuria has an additional interest for us, since in our own experiments the giving of water has occasionally been followed by an intense salivation, a phenomenon which Bernard (1858, pp. 399, 403) not infrequently encountered, doubtless as the result of stimulating the central connections of Wisberg's nerve. In one of our experiments, salivation was noticed an hour after the giving of 250 c.c. water, and its rate gradually increased to a maximum of 41.0 c.c. per 15 minutes, a rate comparable with the mean maximum rate of

urine-flow of this animal, viz., 58 c.c. per 15 minutes. In $2\frac{1}{2}$ hours 140 c.c. of saliva were collected. The urine-flow was unaffected by the giving of the water, and maintained its slow initial rate throughout the experiment. On another occasion* a dog was given a gram of chloralose by mouth, along with 260 c.c. water. The animal passed into a deep sleep, and, 40 minutes after the water had been given, the rate of urine-flow had risen to 37.5 c.c. per 15 minutes. Ten minutes later the rate quickly fell to its initial value, 1.5 c.c. per 15 minutes. salivation began concurrently, and within the next 10 minutes had reached a rate of 30 c.c. per 15 minutes. This high rate continued during the succeeding hour, and 157 c.c. in all were collected. The subcutaneous injection of pituitary extract in a dosage sufficient to arrest a water-diuresis was without influence on the rate of salivary flow (*cf.* Weir, Larson and Rowntree, 1922; Molitor and Pick, 1924). This reciprocity in the secretory activities of kidneys and salivary glands, a not uninteresting example of mobility in biological phenomena, is suggestive again, we feel, of the nervous system's playing some rôle in the sequence of processes initiated by the ingestion of water. But as to whether the polyurias which have been observed to follow severe pain (MacKenzie, 1909, pp. 240-241), cerebral injuries (Kahler, 1886), and cerebral hæmorrhage (Ollivier, 1876) are related, in their underlying causes, with water-diuresis, no evidence is forthcoming upon which a judgment may be based. It would, we feel, be of interest and value, if such polyurias were classified in terms of their responses to pituitary extract.

We have no evidence as to whether the conjectured receptors of the unconditioned stimulus respond to changes in the water-content of the blood rather than of the tissues, though the possibility of eliciting water-diuresis at a time when the tissues contain a large excess of salt (Baird and Haldane, 1922) might incline one to the former view, nor have we evidence as to whether these receptors are centrally or peripherally placed. It is clear, however, from the work of Oehme and Oehme (1918), from that of Weir, Larson and Rowntree (1922), and from our own that neither the renal nor the splanchnic nerves can subserve any such function. Moreover, Janssen's (1928) work excludes the participation of the spinal nerves below the lower cervical region. The participation of the vagus distal to the mid-cervical region is excluded by Motzfeldt's (1917) and Janssen's (1928) observations.

Although the means by which the antidiuretic function of the pituitary body is controlled, are largely, if not entirely, conjectural, a large body of fact

* We are indebted to Dr. G. W. Theobald for this observation.

receives a ready interpretation from, and in turn gives support to, the main theme of the pituitary theory.

From the prolonged physiological responses given by pituitary extracts, it might be tentatively inferred that the maximum degree of inhibition of the gland would not be followed immediately by the maximum response of the kidney. Time is necessary for the normal threshold of activity to fall to a new level, and it is in terms of this delay that we propose to find an explanation of the latency of 15 minutes, which we have already described as existing between the peak of the water-load curve, and the maximum rate of urine-flow. As the water-load curve falls, we may suppose the secretion of the antidiuretic principle to recommence, co-ordination between the water-load and rate of urine-flow being closer during the descent than during the ascent of these curves.

A possible objection to the reality of this delay and its theoretical counterpart, viz., a definite concentration of the antidiuretic principle in the blood stream at a time when the body has just freed itself from an added water-load, is refuted by the facts that, within 15 minutes of the water being given, as much as 105 c.c. has been absorbed, and that, then, the rate of urine-flow has already begun to rise. The animals are not, of course, initially dehydrated to this degree. In fact, as we have already seen, the degree is about 13 c.c., and this would have the effect of postponing the excretion-curve by less than 2 minutes.

In Part I attention was drawn to the frequent and varying disparity in the courses of the responses of the kidneys in any one animal. In order to account for this, we must assume a varying responsiveness by the renal tissues to a fall in the content of the blood in pituitary activity, a responsiveness which may, or may not be conditioned by the rate at which the antidiuretic principle is destroyed after its embodiment in the renal parenchyma. The following observation may have some bearing on the phenomenon. In one of a series of experiments performed by one of us (E. B. V.) to determine the effect on the secretion of the isolated kidney of the passage of blood through the head of the dog (Verney, 1926), a heart-lung-kidney had been prepared, but, owing to the unsatisfactory behaviour of the kidney, the decision was made to substitute for it one from the dog on which hypophysectomy had already been performed, and the characteristic polyuria obtained. Immediately the kidney was connected, a flood-diuresis, about 20 c.c. per 15 minutes, occurred, and persisted till the end of the experiment 2 hours later. We have not repeated this experiment, but the fact that during a fairly long experience of

the properties of the isolated kidney, we have never encountered so immediate and large a response, inclines us to suggest that it is related to a destruction of the antidiuretic principle by the kidney, in the interval of time between hypophysectomy and removal of the kidney to the heart-lung circuit.

The fact, however, that the responses of the two kidneys are not simultaneously evoked, demands the ascription of at least part of the total delay between the absorption and excretion of water, to processes located within the kidney. This must be recognized as a serious objection to the pituitary hypothesis; a not insuperable one, however, since the rate of fall in the concentration of antidiuretic principle within the kidney, can hardly be expected to depend solely upon its rate of fall in the blood stream.

We have seen that the inhibition of water-diuresis by mild exercise, is independent of the nerve-supply to the kidney, is accompanied by a rise in the percentage amount of chloride in the urine, and reaches its maximum degree a few minutes after the exercise has ended. The proximate cause of these changes is, therefore, a humoral one. An interpretation of the results in terms of an increased activity of the suprarenal glands is, we believe, disproved by the fact that bilateral division of the splanchnic nerves, central to the glands, leaves the response unaffected, fig. 2. Further, with respect to the rise in the concentration of chloride, it is of interest that inhibition of the rate of urine-flow, by the addition of adrenalin to the blood in a heart-lung-kidney preparation, is unaccompanied by such rise (Starling and Verney, 1925). The effects of exercise on water-diuresis may be readily interpreted, however, in terms of an increase in the activity of the pituitary body, though it is by no means clear how such an increase may be brought about. The fall in the output of water, and the rise in the percentage amount of urinary chloride, find explanation in a rising concentration of antidiuretic principle in the blood stream, and the progression of the changes observed at the cessation of exercise, in a still further rise, though temporary, in the concentration of antidiuretic principle. This may possibly be owing to the diffusion of an accumulation of principle into the blood, thus delaying the time when the rate of its destruction shall exceed that of its secretion.

We pass now to the consideration, so far as they appear to be susceptible of experimental proof or disproof, of certain consequences which must inevitably flow from the propositions which theory has laid down. These consequences are that a water-diuresis should, *ceteris paribus*, not only follow the intravenous administration of water, but also antecede its normal time of occurrence, when the injection is made at a rate greater than that at which water is normally

absorbed from the gut, or when carefully graded exercise reduces a commencing response to the initial rate of urine-flow. This antecedence should also occur during the ebb of a water-diuresis, and in diabetes insipidus. A further consequence of the theory is that removal of the hypophysis, when the body is optimally hydrated should be succeeded, *ceteris paribus*, by an increased secretion of water by the kidney.

The intravenous injection of water at rates equal to or greater than, that at which it is absorbed from the gut, viz., 7 c.c. per minute in the dog, can hardly be expected to be unaccompanied by disturbances tending to veil any effect which might be ascribed to the presence of the water alone. In absorption from the gut, the local fall in osmotic pressure will be minimized by the enormous surface from which absorption occurs, and by the large flow of blood through the mesenteric vessels. Moreover, any such fall will be rapidly reduced by the high osmotic pressure of the blood in the hepatic veins. Lehmann (1855) detected a reduction to the extent of 25 per cent. in the diameters of the red blood corpuscles during their passage through the liver, and attributed this to glycogenesis (*cf.* Hashimoto, 1914). The anuria, or albuminuria, or hæmaturia, which have been described by various authors to result from the intravenous injection of water (Majendie, 1859; Kierulf, 1853; Hermann, 1859; Falck, 1872; Moutard-Martin and Richet, 1881; Mairet and Bosc, 1892; Bosc and Vedel, 1896; Frey, 1906; Ussner, 1911), are by no means invariably found, especially when care is taken to grade the injection within reasonable limits. Thus Hermann (1859), Westphal (1860) and Bosc and Vedel (1896) all obtained very high rates of urine-flow from intravenously given water. But it is clear, not only that high rates of urinary flow may follow the intravenous administration of water, but also, from Falck's (1872) work, that the maximum rate following such administration, may both exceed that attained as the result of oral administration, and precede it in time. To quote from Falck's paper, "Veranlasst die Wasserinfusion keine Störung, so läuft das Wasser durch die Nieren mit ebenso schneller und unverkennbarer Fluth als wie das dem Magen übergebene Wasser."

With regard to the time of recurrence of a diuresis which has been inhibited by exercise, we have no evidence of value to offer. It is evident that the investigation of this aspect of the general problem is best undertaken on the human subject. We have, however, on several occasions given a second dose of water to a dog before the diuresis from the first has subsided, and have observed the earlier onset which theory demands. Conversely, an increased delay is to be seen when the animal's tissues are dehydrated, a phenomenon

which we suggest is due to the longer time required on the one hand for the water-load to reach the level requisite for inhibition of the pituitary body to occur, and, on the other, for a higher concentration of antidiuretic principle in the blood and kidneys to fall to the level at which the secretion of water is released.

Again, in diabetes insipidus, a response to ingested water should, by hypothesis, occur earlier than in the normal subject. We have made a few observations* on this question, the responses which were compared being obtained from a healthy man *et. 37*, and from a man of the same age but suffering from diabetes insipidus.

The patient N. T. has a 17 years' history of thirst and polyuria. No other abnormal symptoms or signs are found, excepting an increase in weight to a present figure of 94 kg. The man leads an active physical and intellectual life, and his only disability is excessive thirst and polyuria. During the day he drinks about a litre of water every hour, and retires at night with a 2 litre-jug of water by his side. He responds effectively to pituitary extract, but has given up the treatment as he finds the injections more distracting than the complaint itself. The control subject has a gastro-enterostomy, made five years ago; and skiaseopic examination two years ago demonstrated the immediate passage of liquid from stomach to jejunum. It is improbable, therefore, that absorption occurs at a rate slower than in the patient N. T.

The bladders were emptied voluntarily every 5 minutes for the periods immediately preceding and succeeding the drinking of the water. The following figures were obtained:—

Table II.

Normal subject.				Diabetic subject.			
Water drunk c.c.	Period of drinking min.	Period (min.) between mid-time of drinking, and first increase in rate of urine flow.	Remarks.	Water drunk c.c.	Period of drinking min.	Period (min.) between mid-time of drinking, and first increase in rate of urine flow.	Remarks.
1000	3	19	No thirst.	1000	3	15	Great thirst.
2000	9	18	"	2000	1½	12	Thirst.
2000	15	17	"	1160	2	10	No thirst.
				1000	2½	7½	Slight thirst.
				1000	2½	7½	No thirst.
				1000	2½	7½	"

* These observations were made at University College Hospital Medical School under conditions of constant air temperature and humidity, the subjects being stripped of clothing and seated in a special room. (Part I, p. 509).

The possibility of obtaining, by catheterization, a more accurate measurement of the interval between the ingestion of water and the response of the kidney was investigated. Mr. F. J. F. Barrington was kind enough to pass a catheter on a normal subject, and the subject, some time later, drank 1000 c.c. water. The rates of collection were, however, less regular than when voluntary micturition was performed. This was doubtless due to the initiation of reflex contraction of the bladder by small and inevitable movements of the catheter in the posterior urethra (Barrington, 1921). It seems that the most accurate figures in this regard, in man, are to be obtained by careful training of the subjects to frequent and complete emptying of the bladder.

The figures in Table II suggest to us, that the response of the kidney to ingested water is less delayed in a case of diabetes insipidus than in a normal subject. The investigation, however, clearly needs extension before any positive conclusion can be drawn.

The pituitary hypothesis also demands that the dissociation of pituitary from renal function should be followed by an increased output of water by the kidney. We should expect such to follow hypophysectomy, and might anticipate a similar result from excising the kidney and perfusing it in the isolated state. Under both sets of conditions a polyuria of the expected type supervenes. In the former case, however, removal and response, as has been shown by one of us elsewhere (Verney, 1929) are inconstantly conjoined; and as possible factors to which this, and observed differences in the degree of response might be ascribed, were mentioned the depth of anaesthesia, and the age and constitution of the animal. One other point of possible relevance was noted at the time these experiments were performed. The impression was gained that the degree of probability of a polyuria's supervening, was increased when the gland came away cleanly and wholly on applying the forceps to the infundibular stalk. We have no knowledge of the potency of the secretory products of the pituitary body as they leave the site of their formation, and it may well be that piecemeal removal is accompanied by the liberation and absorption of amounts of antidiuretic principle sufficiently large to delay and diminish the expected response. We are inclined to regard, therefore, the resultant polyuria rather as being essentially a manifestation of pituitary removal than as being its accidental accompaniment.

We may now restate the pituitary hypothesis in the following form. The secretion of water, over and above that required for the solutes of the urine, is conditioned by and dependent upon a fall in the concentration in blood and kidney of the antidiuretic principle of the pituitary body. The secretion of the antidiuretic principle is itself controlled, through the intermediation of the nervous system, by the concentration of water in blood and tissues.

Although the pituitary hypothesis gives an intelligible account of water-diuresis and the characters of this phenomenon so far as they are at present known, it is well to realize that it depends fundamentally on the recognition of the essential similarity of water-diuresis to diabetes insipidus and the polyurias following piquêre, experimental removal of the pituitary body, and isolation of the kidney. One cannot fail to perceive the superficial agreement of the corresponding ideas, but lightly to join to them the idea of similar causation is to cast beyond the immediate evidence. Not to realize this would be to confound properties with functions, and so to ignore the feeling of doubt and danger which constantly attends any attempt to apply the method of synthesis to the interpretation of biological events. It is not surprising, therefore, that other hypotheses have been proposed to explain the phenomenon of water-diuresis. These are, first, that the diuresis is conditioned by a hormone absorbed from the intestinal tract by the ingested water (Ginsberg, 1912; Cow, 1912, 1914, *a* and *b*; Ambard and Schmid, 1929); second, that the diuresis is effected through the intermediation of the renal nerves (Fee, 1929, *b*; Bayliss and Fee, 1930); third, that the kidney is responding directly to an increase in the ratio of water-molecules to total molecules in the plasma—the “diffusion-pressure of water” (Haldane and Priestley, 1916; Priestley, 1921; Adolph, 1923, 1925; Beadle and Priestley, 1925).

The conclusion of Ginsberg (1912) and of Cow (1912) being based on evidence of so equivocal a nature, is, in our opinion, quite unjustifiable; and in the later development of the hypothesis by Cow (1914, *a* and *b*) evidence is adduced to show that the increased flow of urine is brought about through *stimulation* of the pituitary body by the intestinal hormone. With Ambard and Schmid the idea to which the term water-diuresis is annexed is so different from the signification which we attach to the term, that it would be idle to attempt to find in their work a bearing on the phenomenon under discussion. Moreover, as has already been pointed out, there is abundant evidence that the kidneys are able to respond by a flood-diuresis to water intravenously administered.

That the renal nerves are functionally involved in the phenomenon of water-diuresis, is an hypothesis brought forward by Fee (1929) and Bayliss and Fee (1930), in interpretation of their experiments on the excretion of urine after hypophysectomy and decerebration, and on the excretion of urine by innervated and denervated kidneys perfused with the heart-lung preparation. They believe the typical polyuria not to occur except through an inhibition of activity in the renal nerves. We have been unable, however, to find clear evidence

in their papers for such a predication of the diuresis following hypophysectomy or isolation of the kidney, and *a fortiori* of that following water-ingestion. Moreover, the evidence collected and produced in Part I of this work proves, as we think, conclusively, that water-diuresis is independent of the presence of the renal nerves.

The hypothesis of Haldane and Priestley must be recognized as at present unassailable. It fails, however, to embrace the remaining polyurias which resemble it so closely, since there can be no reasonable doubt that these are not initiated and maintained by an increase in the concentration of water in the blood. This is evident in the case of the heart-lung-kidney preparation, in which the concentrations of hæmoglobin and serum-chloride may rise well above their normal levels while the polyuria is still in evidence. Again, the polyuria following hypophysectomy in the dog is, as has been shown in unpublished observations by one of us (E. B. V.), accompanied by a perceptible rise in the percentage of hæmoglobin over the figure obtained before hypophysectomy. Those who adhere to the pituitary hypothesis believe an increase in the concentration of water in the blood to be the mediate stimulus to the kidney in water-excretion; those who adhere to the hypothesis of Haldane and Priestley believe it to be the immediate stimulus. Should the latter hypothesis prove to be correct, it follows that the adherents to the former, in causally associating water-diuresis with the polyurias of diabetes insipidus, piqure, isolation of the kidney, and hypophysectomy, are being misled by similitude.

We suggest, therefore, that in the absence of any method which gives to the correlation of water-diuresis and pituitary function the force of demonstration, future work would profitably be directed towards the discovery of similarities and differences in the properties of the polyurias alluded to above -- and the action of thyroxine in increasing the response of the kidney to water, as demonstrated in this paper, might well be made the basis of one such investigation. Until such time, however, as the elicitation of further facts shall demand revision or abandonment of the idea, we propose to regard variations in the secretory activity of the pituitary body and in the output of water by the kidney as forming a conceptual unity.

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IV. *Summary.*

(1) The inhibition of water-diuresis by exercise is independent of the nerve supply to the kidney, the inhibitory responses running closely parallel on the innervated and denervated sides.

The inhibitory response can be readily initiated after functional division of the splanchnic nerves on both sides, and at a time when absorption of water from the gut is complete.

The inhibition, observed at the close of a period of steady and mild exercise, does not reach its full extent until 2 to 14 minutes have elapsed, the minimum rate usually occurring between the third and sixth minute. The percentage of chloride in the urine secreted during the period of inhibition, rises.

(2) The inhibition by post-pituitary extract of an established water-diuresis is independent of any action which the extract might have on the rate of absorption of water by the gut.

When one kidney is denervated and the other not, post-pituitary extract inhibits an induced water-diuresis to the same degree on the two sides, the responses maintaining a close parallelism. This is still true after a period sufficiently long to make it highly probable that complete degeneration of the divided sympathetic fibres has ensued.

(3) The subcutaneous injection of thyroxine increases the intensity, and diminishes the duration, of the response of the kidney to water-ingestion. We are of the opinion that these effects are the expression of a direct action of the drug on the kidney.

(4) Attention is drawn to some further observations, namely, the reciprocity which may sometimes be observed between salivary and urinary secretion of water; the smaller latency between the ingestion of water and the onset of diuresis in a man with diabetes insipidus than in a normal control; and the changes in the percentage of hæmoglobin following hypophysectomy in the dog.

(5) The observations which we have brought forward, along with those of cognate interest derived from other sources, have been considered in the light of the hypothesis which would ascribe the relation of cause and effect to variations in the secretion of an antidiuretic principle by the pituitary gland on the one hand, and variations in the output of water by the kidneys on the other.

The opinion is formed that this hypothesis gives a more fitting interpretation of the facts than one which assumes the kidney to be the definitive seat of the processes responsible for the excretion of water. Moreover, the fertility of the latter hypothesis would appear to suffer reduction by its narrower extension and smaller penetrability.

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